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GENETIC MECHANISMS OF STRESS RESPONSE AND SPORULATION IN *CLOSTRIDIUM BOTULINUM*

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ACADEMIC DISSERTATION

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Cover picture: DNA microarray hybridization of *Clostridium botulinum*

ABSTRACT

Clostridium botulinum presents a significant hazard to the food processing industry. However, the cellular mechanisms and factors that contribute to their regulation utilized by this feared foodborne pathogen in response and adaptation to food processing and storage-induced stress are poorly characterized. Another major aspect of *C. botulinum* presenting serious implications on food safety is its capability to produce heat-resistant endospores. Nevertheless, the sporulation cascade of *C. botulinum* has not been characterized. This study sought to investigate the effects of temperature downshift on the global gene expression pattern of Group I *C. botulinum* type strain ATCC 3502, and further characterize the roles of regulatory mechanisms identified as cold tolerance-related. Furthermore, the role of a major regulatory element, the alternative sigma factor SigK, in the sporulation cascade of *C. botulinum* was determined. Additionally, its putative function in stress tolerance was investigated.

Transcriptomic analysis of the foodborne pathogen *C. botulinum* ATCC 3502 upon temperature downshift revealed the induction of several mechanisms previously identified as cold-related in other bacteria, thus suggesting that also *C. botulinum* utilizes these mechanisms in cold tolerance. Mechanisms with hitherto uncharacterized functions in cold tolerance were also found. The results suggested that secondary oxidative stress was present as a component of cold stress. Additionally, two previously uncharacterized putative DNA-binding regulatory proteins CBO0477 and CBO0558A were shown to play a role in cold tolerance of *C. botulinum* ATCC 3502.

The two-component system (TCS) CBO0366/CBO0365 was shown to be important in the cold tolerance of *C. botulinum* ATCC 3502. Expression of this TCS was induced upon temperature downshift, but not under optimal temperature and growth conditions. Disruption of either of the TCS components resulted in deteriorated cold tolerance, whereas over-expression of *cbo0366* in a wild-type strain resulted in an increase of growth rate at low temperature.

Inactivation of the TCS response regulator-encoding *cbo0365* markedly altered the transcriptome of *C. botulinum* ATCC 3502. Totals of 150 and 141 chromosomal coding sequences (CDS) were significantly differently expressed in the *cbo0365* mutant at either 37 °C or 15 °C, respectively. There was an overlap of 141 common CDSs between the two temperatures. The genes differentially expressed included ones related to acetone-butanol-ethanol (ABE) fermantion, arsenic resistance, phosphate uptake and flagellar rotation. The involvement of CBO0365-regulated metabolic pathways in cold tolerance was demonstrated by the deteriorated cold tolerance of mutants of the respective pathways. Cold-sensitive phenotypes were observed for

mutants of the acetone-butanol-ethanol fermentation pathway components *bcd*, *crt*, *bdh* and *ctfA*, the arsenic detoxifying machinery components *arsC* and *arsR*, and the phosphate uptake mechanism component *phoT*. Electrophoretic mobility shift assays confirmed transcriptional activation or repression as a means for CBO0365 in regulating itself, and the *crt, ars*, and *pho* operons.

A dual role for the alternative sigma factor SigK in sporulation and in stress tolerance of *C. botulinum* ATCC 3502 was demonstrated. Disruption of SigK halted sporulation at an early stage but the phenotype was restored by *in trans* complementation of the mutation. The expression of *sigK* was induced upon exposure to low temperature and high salinity, but not upon a downshift in pH. A deteriorated tolerance to low temperature and to high salinity was observed for the *sigK* mutant strains.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are hereafter referred to in the text by their Roman numerals:

I Dahlsten, E., Isokallio, M., Somervuo, P., Lindström, M., & Korkeala, H. (2013) Transcriptomic analysis of (Group I) *Clostridium botulinum* ATCC 3502 cold shock response. Submitted for publication.

II Lindström, M., Dahlsten, E., Söderholm, H., Selby, K., Somervuo, P., & Korkeala, H. (2012) Involvement of two-component system CBO0366/CBO0365 in the cold shock response and growth of group I (proteolytic) *Clostridium botulinum* ATCC 3502 at low temperatures. *Appl Environ Microbiol.* **78**:5466-70.

III Dahlsten, E., Zhang, Z., Somervuo, P., Minton, N. P., Lindström, M., & Korkeala, H. (2014) The cold-induced two-component system CBO0366/CBO0365 regulates metabolic pathways with novel roles in cold tolerance of Group I *Clostridium botulinum* ATCC 3502. *Appl Environ Microbiol.* In press.

IV Kirk, D., Dahlsten, E., Zhang, Z., Korkeala, H., & Lindström, M. (2012) Involvement of *Clostridium botulinum* ATCC 3502 sigma factor K in early-stage sporulation. *Appl Environ Microbiol.* **78**:4590-6.

V Dahlsten, E., Kirk, D., Lindström, M., & Korkeala, H. (2013) Alternative sigma factor SigK has a role in stress tolerance of Group I *Clostridium botulinum* strain ATCC 3502. *Appl Environ Microbiol.* **79**:3867-9.

ABBREVIATIONS

ABE	Acetone-butanol-ethanol
ATCC	American Type Culture Collection
a_w	Water activity
BCFA	Branched-chain fatty acid
BoNT	Botulinum neurotoxin
bp	Base pair
cDNA	Complementary DNA
CDS	Coding sequence
Cq	Quantification cycle
CSP	Cold shock protein
EMSA	Electrophoretic mobility shift assay
FA	Fatty acid
FDR	False discovery rate
HK	Histidine kinase of two-component signal transduction system
HSP	Heat shock protein
LB	Luria Bertani
OD_{600}	Optical density at 600 nm
ORF	Open reading frame
PCR	Polymerase chain reaction
RR	Response regulator of two-component signal transduction system
RT	Reverse transcription
RTE	Ready-to-eat
RT-qPCR	Quantitative reverse-transcription real-time polymerase chain reaction
TCS	Two-component signal transduction system
TPGY	Tryptone-peptone-glucose-yeast extract
UV	Ultraviolet light

1. INTRODUCTION

Over two centuries ago, reports of increasing numbers of poverty-stricken Germans succumbing to a fatal paralytic illness were made. The aftermath of numerous wars had resulted in an overall decline of hygienic standards in food preparation. Initial speculations connected the outbreaks to the consumption of traditional smoked blood sausages. The first systematic review of the symptoms of "sausage poisoning" was published in 1817 by the German physician Justinus Kerner (Kerner, 1817). The disease was named "botulism" after the Latin word for sausage "*botulus*". In addition to his thorough, accurate description of botulism symptoms that are still valid today, Kerner amazingly was the first to recognize the potential therapeutic uses for the botulinum neurotoxin, or, "sausage poison". Kerner's prescience in this regard was not acted upon until nearly two hundred years later. Advancing knowledge of microbiology methods eventually enabled the Belgian microbiologist Emile van Ermengem to isolate an anaerobic bacillus. This bacillus was associated to a fatal botulism outbreak that resulted from the ingestion of home-cured ham at a funeral wake (van Ermengem, 1897). The organism was called *Bacillus botulinus*, and was later renamed to *Clostridium botulinum*.

After the initial epidemiological linking of botulism to undercooked meat, the fact that the causative organism was not directly associated to meat *per se*, but instead was widely dispersed in nature became to be understood and accepted (Burke, 1919). Later on, the increasing knowledge of basic physiological characteristics of *C. botulinum* allowed the identification of food safety risks presented by the organism, and consequent candidate control measures were put forward to reduce these risks (Thom, 1922). Important aspects in the control of *C. botulinum* in foods, i.e. its ability to form endospores that have significant resistance to food processing treatments, the requirement for an anaerobic environment for growth and toxin production, and the heat-lability of the extremely potent toxin, had already been elucidated to a certain extent. However, further characterization of the organism revealed several alarming phenotypic properties. A phenotypically distinct group of *C. botulinum* strains was found to be able to grow at refrigeration temperatures (Eklund *et al.*, 1967). Moreover, these strains lacked proteolytic enzyme activity, thus the sensory qualities of foods could remain acceptable to the consumer while botulinum toxin was already being produced in potentially lethal quantities.

The underprocessing of "ready-to-eat" products of Kerner's era was the result of a lack of knowledge and poor facilities, whereas the modern-day consumer presents a conscious demand to the food industry that his or her quick meal would be processed as little as possible. Attempts to develop processes that meet such demands, while implementing sufficient measures

to ensure food safety, is a considerable challenge (Peck, 2006). A concept of "hurdles" was defined for implemented measures to control or eliminate pathogenic microorganisms in foods. The basic idea of hurdles is that multiple preventive measures aggregate to present an impassable blockade to the pathogen, while the individual treatments by themselves remain relatively mild (Leistner, 2000).

A sufficient reduction in the number of heat-resistant spores of *C. botulinum* to attain a level whereby the spores no longer present a hazard regardless of further hurdles requires harsh heat treatment. Such harsh treatment by definition would be unacceptable when minimal processing is demanded by the consumer. Therefore, the remaining obstacles must provide enough of a barrier for *C. botulinum* outgrowth and proliferation to ensure the safety of the food product after a potential shelf-life of several weeks (Peck, 1997; Lindström *et al.*, 2006). The foremost of these hurdles is an uninterrupted chill storage of the food. A profound understanding of the genetic mechanisms foodborne pathogenic bacteria activate upon cold stress is therefore of paramount importance in the planning and implementation of adequate food safety control measures.

While considerable amounts of data on the specific and global mechanisms various pathogenic and non-pathogenic microorganisms utilize in countering cold stress have recently become available (Phadtare, 2004), only a few reports address the cold-tolerance mechanisms of *C. botulinum* (Söderholm *et al.*, 2011; Derman *et al.*, 2013a). The precise and rapid regulation of gene expression activated by specific environmental factors is crucial in triggering the adaptive response to the situation in hand (Price, 2011). Therefore, the cold stress response mechanisms and especially their regulatory elements in *C. botulinum* require thorough investigation. Furthermore, a food safety hazard of substantial interest arises from the considerable resistance of *C. botulinum* spores towards treatments targeted in their inactivation. While the knowledge of the sporulation pathways and their regulation is substantial in the model sporeformer, *Bacillus subtilis* (Piggot & Hilbert, 2004), the mechanisms of spore formation in *C. botulinum* have not been elucidated. A thorough characterization of the sporulation mechanisms of *C. botulinum* and their putative interconnections to stress tolerance should therefore be carried out.

2. REVIEW OF THE LITERATURE

2.1 *Clostridium botulinum* and botulism

2.1.1 *Clostridium botulinum*

Clostridium botulinum is a strictly anaerobic, Gram-positive rod-shaped bacterium, which is able to form endospores that are highly resistant to harsh environmental conditions. The bacterium also produces the most potent biological toxin known to man, botulinum neurotoxin. *C. botulinum* strains are divided into four groups (denoted I-IV) according to their physiological characteristics, and into eight serotypes (denoted A-H) according to the antigenic properties of the specific botulinum neurotoxins that are produced by the strain (Cato *et al.*, 1986; Collins *et al.*, 1994; Collins & East, 1998; Barash & Arnon, 2013). Of strains characterized to date, Group I strains produce neurotoxin serotypes A, B and/or F, or B and H, Group II strains types B, E or F, Group III strains types C or D, and Group IV strains type G (Hauschild, 1990; Barash & Arnon, 2013; Dover *et al.*, 2013). The strains of Groups I and II produce toxins that are associated with human disease, whereas toxins produced by group III strains cause animal botulism (Lindström *et al.*, 2004; Myllykoski *et al.*, 2009; Myllykoski *et al.*, 2011). The neurotoxin type G produced by the rare Group IV strains has not been associated with disease either in humans or animals, albeit classical botulism signs have manifested in animals when inoculated with type G neurotoxin (Ciccarelli *et al.*, 1977). Group IV strains were suggested to be renamed as *Clostridium argentinense* (Suen *et al.*, 1988a). Additionally, several dual-toxin producing strains, and strains that harbor a silent neurotoxin gene cluster have been identified (Franciosa *et al.*, 1994; Carter *et al.*, 2009; Barash & Arnon, 2013).

Classification of a bacterial strain as belonging to the *C. botulinum* species still relies on that strain's ability to produce botulinum neurotoxin (Cato *et al.*, 1986). However, this approach has been deemed inappropriate on several occasions, as the strains belonging to different groups have distinct phenotypic (Lynt *et al.*, 1982; Cato *et al.*, 1986) and genotypic characteristics (Collins *et al.*, 1994; Collins & East, 1998; Hielm *et al.*, 1999; Hyytiä *et al.*, 1999; Keto-Timonen *et al.*, 2005; Hill *et al.*, 2007; Carter *et al.*, 2009; Peck, 2009). Furthermore, strains of different phenotypic groups have been suggested to belong to entirely different phylogenetic lineages (Collins & East, 1998; Keto-Timonen *et al.*, 2005). The taxonomic classification of *C. botulinum* is further complicated by the ability of some strains of *Clostridium butyricum* and *Clostridium baratii* to produce botulinum neurotoxin types E and F, respectively (Suen *et al.*, 1988b). Despite all these

anomalies, the classical nomenclature is still retained to avoid confusion in clinical practice.

Group I *C. botulinum* strains are mesophilic, their optimal growth temperature is commonly reported as 37 °C albeit some variation occurs (Hinderink *et al.*, 2009). The minimum and maximum growth-limiting temperatures are classically reported to be 10 °C and 50 °C (Lynt *et al.*, 1982). A significant degree of variation in the growth boundaries was recently reported, whereby the minimum growth temperature varied between 12.8 °C and 16.5 °C, and the maximum varied between 40.9 °C and 48.0 °C (Hinderink *et al.*, 2009). The strains' abilities to grow are also limited by a water activity value (a_w) of 0.94, which corresponds to 10% w/v NaCl in brine (Hauschild, 1989). No growth for Group I strains has been observed in foods where the pH is <4.6 (Raatjes & Smelt, 1979; Smelt *et al.*, 1982; Smith & Sugiyama, 1988; Hauschild, 1989). The Group I strains are able to produce endospores with a considerably high heat resistance.

Strains belonging to Group II differ substantially from their Group I counterparts in their physiological characteristics. In contrast to the mesophilic Group I strains, Group II strains are psychrotrophic, i.e. their optimal growth temperature is commonly reported as 25 °C (Smith & Sugiyama, 1988; Hauschild, 1989). However, increased growth rates at 37 °C compared to 30 °C have been observed for several Group II strains (Derman *et al.*, 2011). Importantly, several Group II strains have been reported to grow and produce toxin in culture media over a course of 5 to 6 weeks at a temperature as low as 2.9 °C (Graham *et al.*, 1997), and in foods at 8 °C within a 12-day period (Betts & Gaze, 1995). These phenotypic characteristics present a considerable risk in minimally processed, ready-to-eat (RTE) foods with extended shelf lives (Peck, 1997; Lindström *et al.*, 2006; Peck, 2009; Peck *et al.*, 2011). In contrast to Group I, Group II strains are considerably less robust to low pH or the inhibitory effects of low water activity (Smith & Sugiyama, 1988; Graham *et al.*, 1997). Moreover, Group II spores are less resistant to heat treatment than Group I spores (Smith & Sugiyama, 1988; Stringer *et al.*, 1997; Lindström *et al.*, 2006).

Group III *C. botulinum* strains are mesophilic, with an optimum growth temperature of 40 °C (Smith & Sugiyama, 1988). These strains have limited resistance to low pH (growth-limiting pH of 5.1 to 5.6) and high NaCl (growth-limiting brine concentration 2.5% w/v) (Segner *et al.*, 1971). Group III spores manifest an intermediate resistance to heat.

Differentation between *C. botulinum* Group I and II strains is of considerable importance in determining the possible source of a contamination in an outbreak (Lindström & Korkeala, 2006). However, such a determination can not readily be achieved by toxin type determination alone. Such an approach is only feasible for toxin type A or E strains, as these toxin types are to date considered as restricted to Group I or Group II strains, respectively (Hauschild, 1990; Peck *et al.*, 2011). Identification of a strain as Group I or II has conventionally been achieved by monitoring the strain's

proteolytic activity on a casein-based agar. Several molecular biology typing methods have been shown to be able to differentiate the strains into groups (Hutson *et al.*, 1993; Hielm *et al.*, 1999; Keto-Timonen *et al.*, 2005; Hill *et al.*, 2007). Furthermore, a simple PCR-based assay was recently developed to accomplish this task (Dahlsten *et al.*, 2008).

2.1.2 Botulism

Botulinum neurotoxin

The causative agent of botulism, botulinum neurotoxin (BoNT), is the most potent biological substance known. Nanogram quantities of the compound have been calculated to be sufficient to cause potentially fatal intoxication in adult humans (Peck, 2009). The extreme potency of the toxin has aroused concern about its potential as a biological warfare agent (Arnon *et al.*, 2001). However, despite this ill repute, the pharmaceutical industry has adopted the toxin for use in the treatment of several neuromuscular disorders. Furthermore, the use of purified botulinum neurotoxin preparations in cosmetics is widespread. The specific molecular target of the toxin varies depending on the toxin type. However, BoNT intoxication ultimately results in the blockade of the neuromuscular signal transmission that leads to progressive flaccid paralysis of muscle. Although some effects to the central nervous system have been proposed (Drinovac *et al.*, 2013), cognitive functions have been considered unaffected by the toxin.

The active neurotoxin is a zinc-dependent metalloendopeptidase, with a toxin type-specific affinity to different components of the neuroexocytosis mechanisms of humans and animals. Notwithstanding their different specificities, all toxin types cleave an essential component of the acetylcholine secretion mechanism, which results in the blockade of acetylcholine secretion from the motor nerve ending. This, in turn, inactivates the signal transduction from the nerve to the muscle (Rossetto *et al.*, 2006). Recently, BoNTs have been shown to have significant effects in treating severe neurogenic pain (Jabbari & Machado, 2011). This suggests additional functionality for the toxin apart from from those classically described. It was recently proposed, that neuroexocytosis mechanisms, other than from acetylcholine secretion, might also be targets for the neurotoxin molecule (Aoki, 2005). Alternatively, the analgesic effect of BoNT is proposed to be mediated directly via the central nervous system μ-receptor activation and the subsequent release of endogenous opioids (Drinovac *et al.*, 2013).

Regardless of the toxin type, the BoNT molecule is ribosomally synthesised during late exponential growth as an approximately 150-kDa single-chain polypeptide. In order to activate its neurotoxic potential, the single-chain toxin molecule undergoes proteolytic activation, “nicking”, where the polypeptide chain is cleaved, which results in the formation of a

dichain molecule linked by a disulphide bridge (DasGupta, 1989). In Group I (proteolytic) *C. botulinum*, the cleavage is accomplished by the organism's own proteolytic enzymes (DasGupta, 1989). In contrast, the (nonproteolytic) Group II strains rely on the digestive enzymes of the host's gut to activate the toxin. The toxin molecules are heat-labile: therefore heating at 85 °C for 5 min is generally considered sufficient for inactivation of the toxins (Siegel, 1993). The toxin complex may harbour several additional nontoxic components; the organization of which depends on the strain and toxin type (Hill *et al.*, 2009). The exact roles of these additional components remain to be fully characterized. However, they are suggested to protect the toxin molecule from the harsh conditions of the gut (Gu *et al.*, 2012; Benefield *et al.*, 2013), and possibly also facilitate uptake of the toxin from the intestinal lumen (Fujinaga *et al.*, 1997; Matsumura *et al.*, 2008; Fujinaga, 2010). Botulinum neurotoxin must enter the bloodstream in order for it to reach its targets, i.e. the peripheral motor nerve endings. The entry mechanisms are obvious in the case of *in vivo* toxin formation in deep wounds, or in the cases of maladministration of therapeutically or cosmetically intended toxin preparations. In contrast, the mechanisms by which the toxin can enter the circulation from the intestine are not clear.

The disease

The severe neuroparalytic disease of mammals and avian species resulting from intoxication by botulinum neurotoxin is called botulism. The disease in humans proceeds as a symmetric descending flaccid paralysis, beginning from the facial musculature that is innervated by the cranial nerves. Initial symptoms and signs thus present as the result of weakness of these muscles as manifested by difficulty in speech and swallowing. Double or blurred vision is commonly reported. Dry mouth and sore throat are other commonly described early symptoms. The paralysis then progresses to the more peripheral parts of the body, subsequently rendering the patient nonambulatory. Finally, the respiratory muscles are affected, and if left untreated at this advanced stage, botulism can ultimately result in death due to paralysis of the respiratory muscles (Sobel & Maslanka, 2012).

Human botulism can be divided into noninfectious (intoxication) and infectious types. The classical intoxication form of botulism is foodborne, commonly referred only as "classical botulism". In foodborne botulism, the spores present in the food raw materials are not destroyed by the food processing, and subsequently, inadequacy of intrinsic and/or extrinsic factors present in the food allows the germination and outgrowth of spores, and ultimately neurotoxin production by the vegetative bacteria ensues. The intial indications of foodborne botulinum toxin intoxication are typical gastrointestinal food poisoning symptoms, i.e. nausea, vomiting, abdominal pains and cramps, and constipation (Sobel & Maslanka, 2012). The incubation period before the first signs of the disease appear depends on the amount of toxin ingested. Typically this ranges from 18 to 72 hours.

Foodborne botulism associated with Group I *C. botulinum* has been classically linked to improper home preservation practices. However, several recent outbreaks have been attributed to commercially-produced foods (Peck, 2006). Outbreaks of botulism caused by Group II organisms are usually linked to fermented, salted or hot-smoked marine products (Korkeala *et al.*, 1998; Lindström *et al.*, 2006; Peck, 2006; Leclair *et al.*, 2013b). The processes utilized in the preparation of these foods are by definition insufficient to inactivate the Group II spores (Stringer *et al.*, 1997; Lindström *et al.*, 2006) that are commonly found in the raw materials (Craig *et al.*, 1968; Hielm *et al.*, 1998; Leclair *et al.*, 2013a). Subsequent temperature abuse allows the outgrowth and toxin production by Group II *C. botulinum* (Graham *et al.*, 1996; Graham *et al.*, 1997; Peck, 1997; Lindström *et al.*, 2006).

Foodborne botulism is the most common form of botulism. In 1999-2000, more than 2500 cases were reported in Europe (Peck, 2006). Several former Soviet Union countries, and also Poland and Turkey, have high incidence rates (Varma *et al.*, 2004; Peck, 2006). The highest reported national incidence rate of foodborne botulism (0.9/100 000) was reported for the Republic of Georgia where the preparation of homemade canned vegetables were attributed to the majority of cases (Varma *et al.*, 2004).

The most common human infectious botulism type is infant botulism (Midura & Arnon, 1976). The conditions of the human gut appear otherwise favorable for Group I *C. botulinum*, but being a poor competitor, *C. botulinum* outgrowth is normally prevented by the massive amounts of competing microorganisms. However, the naïve intestinal microbiota of infants less than one year old is insufficient to outcompete *C. botulinum*. An epidemiological link with honey has been established for infant botulism as honey is known to contain substantial amounts of *C. botulinum* spores (Nevas *et al.*, 2005a). Feeding honey to infants is therefore universally discouraged by health authorities. However, fewer than 5% of infantile botulism cases in the United States are epidemiologically linked to honey (Koepke *et al.*, 2008). In those cases without a history of honey consumption, the illness was presumed to result from the ingestion of spores from the environment, such as via household dust (Nevas *et al.*, 2005c; Koepke *et al.*, 2008; Derman *et al.*, 2013b). In addition to Group I *C. botulinum*, several cases of infant botulism caused by toxin-producing *C. butyricum* and *C. baratii* (Aureli *et al.*, 1986; Suen *et al.*, 1988b; Fenicia *et al.*, 2002; Barash *et al.*, 2005; Grant *et al.*, 2013) and by the psychrotropic Group II strains (Luquez *et al.*, 2010) have been described. Interestingly, three recent infant botulism cases caused by *C. butyricum* have been associated to pet turtles (Grant *et al.*, 2013). Finally, a single case of infant botulism from type C toxin has been reported (Oguma *et al.*, 1990).

Another type of infectious botulism is wound botulism, whereby spores of *C. botulinum* colonize deep wounds and toxin formation occurs *in vivo*. Although rare amongst the general population, wound botulism incidence

among intravenous drug users is considerable (Brett *et al.*, 2004; Akbulut *et al.*, 2005; Artin *et al.*, 2007). The use of contaminated needles frequently cause abscess formation, which provides sufficiently shielded, anaerobic conditions for the germination and outgrowth from spores (MacDonald *et al.*, 1985). Finally, an extremely rare intestinal infectious form of botulism similar to infant botulism has been described in adults. Most of these cases have a history associated with procedures and treatments that severely disturbed the gut microbiota, such as massive abdominal surgery or prolonged antibiotic treatment. Such interventions significantly reduce the microbial competition and subsequently allow *in vivo* growth and toxin production by *C. botulinum* (Chia *et al.*, 1986; McCroskey & Hatheway, 1988).

Two other noninfectious types of botulism are inhalation botulism, whereby an aerosol of the toxin is adsorbed through the mucous membranes; and iatrogenic botulism, by which therapeutically or cosmetically-indented botulinum toxin is maladministred. Inhalation botulism has been described only once (Holzer, 1962). However, the potential for the dissemination of botulinum toxin as an aerosol for malicious purposes has aroused significant concern (Arnon *et al.*, 2001). Iatrogenic botulism can result from the unintentional maladministration of therapeutic toxin preparations (Mezaki *et al.*, 1996; Partikian & Mitchell, 2007), or from illicit cosmetic use of non-pharmaceutical grade toxin preparations. Concentrations of the latter can significantly vary and are usually considerably higher than those in legitimately prepared products (Chertow *et al.*, 2006).

Treatment of botulism is supportive: most importantly, the respiratory system needs to be artificially supported during the recovery period. The effect of the toxin on the motor nerve endings is irreversible; therefore, the recovery from botulism takes place only through the growth of new axon fibers that replace the damaged motor nerve terminals. Antisera for the toxins are available; however, appearance of neurological symptoms indicates that much of the circulating toxin will already have reached its target tissue, the nerve endings. Thus, the administration of antiserum only inhibits further progression of the disease. Furthermore, the use of the equine-raised antitoxin is linked to severe adverse effects such as anaphylaxis. However, administration of human-derived antitoxin (BIG-IV) for the treatment of infant botulism has been shown to significantly decrease the length of hospitalization and required intensive care (Arnon *et al.*, 2006).

BoNT gene cluster and neurotoxin gene expression

The genes responsible for BoNT production are found in a cluster across different *C. botulinum* strains with the general consensus of them occurring in two major arrangements, the “ha cluster” and the “orf-X cluster” (Peck *et al.*, 2011). These gene clusters consist of two independently-transcribed operons, the *ntnh-bont* operon, and the *ha* (ha cluster) or *orfX* (orf-X cluster) operon (Hill *et al.*, 2009). The *ntnh-bont* operon harbours the genes

that encode the botulinum neurotoxin itself, and a non-toxic non-hemagglutinating component of the toxin complex. An additional gene with unknown function, *p47,* precedes the *ntnh-bont* genes in the "orf-X" arrangement (Hill *et al.*, 2009). The *ha* operon consists of genes that encode three additional neurotoxin complex proteins. In contrast to the *ha* operon, clusters with the "orf-X" arrangement harbour three genes with unknown function (*orfX1-3*). Located between these two operons, is a gene that encodes an alternative RNA polymerase sigma factor, *botR,* that acts as a direct positive regulator of neurotoxin synthesis (Marvaud *et al.*, 1998; Raffestin *et al.*, 2005). Notably, this gene is missing from strains of Group II type E (Hill *et al.*, 2009) and Group II type F (Dover *et al.*, 2011). Considerable diversity of the botulinum neurotoxin gene cluster, without any associations to other known phylogenetic relationship between strains, has been demonstrated (Carter *et al.*, 2009). Furthermore, independent evolution of the neurotoxin locus and frequent horizontal transfers and recombinations of the neurotoxin genes were suggested (Carter *et al.*, 2009; Hill *et al.*, 2009). In addition to the classification into eight toxinotypes (A-H), the following subtypes of BoNTs have been discovered to date: five subtypes of type A (A1 to A5) (Hill *et al.*, 2007; Dover *et al.*, 2009), seven subtypes of type B (B1 to B7) (Kalb *et al.*, 2012), nine subtypes of type E (E1 to E9) (Chen *et al.*, 2007; Hill *et al.*, 2007; Macdonald *et al.*, 2011; Raphael *et al.*, 2012), and seven subtypes of type F (F1 to F7) (Raphael *et al.*, 2010). Although the differences between the amino acid sequences of different subtypes are small compared to the differences between the eight main serotypes, significant differences in antigenic properties have been observed between the subtypes (Smith *et al.*, 2005; Arndt *et al.*, 2006; Smith, 2009; Dover *et al.*, 2013).

The expression of the neurotoxin locus peaks close to the time of entry into the stationary growth phase of *C. botulinum* (Lövenklev *et al.*, 2004; Couesnon *et al.*, 2006; Chen *et al.*, 2008), and then slowly returns to basal levels after 24 h of growth. The onset of BoNT production coincides with the upregulation of neurotoxin gene expression. The highest neurotoxin titers have been measured after 24 h of growth, and the toxin concentration will remain stable for five days (Couesnon *et al.*, 2006). Bacteria utilize so-called "quorum sensing" mechanisms to gather information about the number of their siblings in their vicinity. Induction of BoNT production appears to be growth-phase dependent, i.e. affected by the number of vegetative cells nearby. Therefore, the roles of the putative quorum sensing mechanisms of a Group I type A strain in toxin production were investigated. In that investigation the quorum sensing mechanism *agr-2* was suggested to regulate toxin production positively (Cooksley *et al.*, 2010).

Growth temperature has been shown to affect neurotoxin production and stability. Stable and prolonged neurotoxin gene expression has been observed at refrigeration temperature (10 °C) in a Group II type E strain (Chen *et al.*, 2008). On the other hand, incubation at 44 °C was shown to

activate a BoNT-degrading protease in a Group I type A strain, but not in a Group II type E strain (Couesnon *et al.*, 2006). Other environmental factors identified as influencing BoNT production include carbon dioxide, which enhances toxin production in several Group II strains (Lövenklev *et al.*, 2004; Artin *et al.*, 2008), but not in Group I strains (Artin *et al.*, 2010). Furthermore, the presence of carbon and nitrogen sources in culture media is important for toxin production by Group I strains, whereas high levels of arginine repress toxin production (Patterson-Curtis & Johnson, 1989).

Transcriptional regulation of toxin production appears to be an extremely complex network system with several positive and negative regulatory elements (Connan *et al.*, 2013). The alternative sigma factor BotR has a key role in positively regulating toxin gene expression (Raffestin *et al.*, 2005). Although the Group II type E strains do not harbor a *botR* homologue in their genome, the expression of the neurotoxin genes does follow the same growth phase-dependent pattern observed for *botR*-containing strains (Couesnon *et al.*, 2006). This finding suggests that other key regulatory factors may be present. The TCS, CBO0787/CBO0786, was recently shown to regulate neurotoxin gene expression directly and negatively by inhibiting BotR-mediated transcription of the neurotoxin genes via the blocking of the *ntnh-bont* and the *ha* promoters (Zhang *et al.*, 2013). This TCS is the only negative regulator of BoNT expression as yet identified. Finally, silencing of three two-component system (TCS) response regulator-encoding genes has been shown to reduce neurotoxin production in a Group I type A strain (Connan *et al.*, 2012).

2.2 Effects of environmental stress on bacteria

2.2.1 Environmental stress factors encountered in the food chain by bacteria

The prevention of growth of spoilage organisms and more importantly, pathogens, is a constant challenge in the development of food processing and storage practices (Leistner, 2000). The demands of consumers are increasingly inclined towards minimally processed foods. Furthermore, the use of additives such as preservative agents faces considerable consumer disapproval and resistance. The use of salt, an essential preservative agent in several foods, is also discouraged due to health concerns. Attempts to provide foods as “pure” and minimally processed as possible, while being able to ensure the safety and adequate shelf life of these products are a considerable challenge (Peck, 1997; Lindström *et al.*, 2006; Peck, 2006).

Heating is a central measure in the preparation of RTE foods that imposes a severe stress for any vegetative bacteria present. Importantly, the function and structure of the cytoplasmic membrane of vegetative bacteria are compromised, and the function of proteins is hampered due to denaturation

and misfolding (Guisbert *et al.*, 2008; Klinkert & Narberhaus, 2009). However, minimal heating processes used in order to kill vegetative bacteria are generally insufficient to destroy the spores of sporeforming bacteria present in the foods (Peck, 1997; Stringer *et al.*, 1997; Lindström *et al.*, 2006; Peck, 2006). Importantly, the presence of lysozyme in food allows the germination of heat-stressed spores of Group II *C. botulinum* (Peck *et al.*, 1992a; Peck *et al.*, 1992b; Peck *et al.*, 1993; Lindström *et al.*, 2003). This presents a significant hygiene challenge in the production of safe miminally processed foods, as lysozyme is naturally present in a number of foods (Peck & Stringer, 2005; Peck *et al.*, 2011).

Although the heat treatments utilized in minimal food processing may be sufficient to destroy vegetative bacteria, sporeformers are mostly unaffected (Lindström *et al.*, 2006; Peck, 2006). Therefore, rapid cooling and subsequent stable low storage temperature is the central food safety control measure in minimally processed foods (Peck, 1997; Lindström *et al.*, 2006; Peck, 2006; Peck, 2009). Temperature downshift imposes several challenges to the bacterial cell. Protein synthesis in the cell is impaired due to hampered ribosome function. The high degree of secondary structure formation in messenger RNA molecules under low temperature conditions further hinders protein synthesis (Inouye & Phadtare, 2004; Phadtare, 2004). Importantly, low temperature has a significant deletorious effect on the cytoplasmic membrane of the cell (Suutari & Laakso, 1994). The membrane comprises a lipid fatty acid bilayer, which plays an active role in the cellular metabolism. Temperature downshift causes the high melting-point fatty acids present in the membrane to solidify, thus significantly hampering the functionality of the membrane (Suutari & Laakso, 1994).

Other stress factors encountered by bacteria in the food chain are primarily related to intrinsic factors of the food. Desiccation or increase of solute, i.e. salt and/or sugar, concentration results in decreased a_w and thus hyperosmotic stress in the cells. Acidic conditions (low pH) damage the cell membrane, and have negative effects on several glycolytic enzymes. Furthermore, the membrane gradient is disturbed (Cotter & Hill, 2003). A low pH in foods can be achieved naturally by the endogenous production of acids that result from fermentation processes, or by directly exogenously adding acids to foods as in pickling.

2.2.2 Response of bacteria to food processing-induced stress

The mechanisms that bacteria activate upon environmental stress are widely studied. Classically, a “general” stress response is understood to be activated upon the imposition of any stress condition, and this response provides protection against multiple stressors. In many Gram-positive bacteria, the universal stress response is mediated by the activation of the alternative sigma factor, SigB, through a phosphorelay mechanism with considerable variability between species (Price, 2011). Activation of SigB results in a

dramatic remodeling of the transcriptome (Volker *et al.*, 1999; Brigulla *et al.*, 2003; Hecker *et al.*, 2007). The induction kinetics of specific versus general stress response by SigB has been shown to depend on the rate of the environmental stress imposed (Young *et al.*, 2013). Specifically, a rapid exposure to stress induces a broad, general stress response profile, whereas slowly-imposed stress elicits a more specific response. Evidence for the regulation, or even the presence, of a homologous universal stress response in bacteria that evidently do not harbor a homologue for *sigB* in their genome, has not been reported to date. Moreover, there is no evidence of a *sigB* homologue being present in the genome of *C. botulinum* ATCC 3502 (Sebaihia *et al.*, 2007).

The cold shock response

Upon cold shock, bacteria elicit a targeted set of measures to counter the cold-induced deterioration effects described above. A period of growth arrest usually ensues that allows the bacterial cell to adapt. During this period, the central metabolism and synthesis of vegetative growth-associated proteins are shut down. Synthesis of so-called cold shock proteins (CSPs) can be observed in several bacteria upon temperature downshift (Phadtare, 2004). The cold-related CSPs are understood to assist translation by dismantling the RNA secondary structures (Jiang *et al.*, 1997), and to function as transcription antiterminators (Bae *et al.*, 2000). CSPs are conserved across different kingdoms (Graumann & Marahiel, 1998). The roles of CSPs in cold tolerance of several foodborne pathogens were demonstrated (Wouters *et al.*, 2000), including those in Group I *C. botulinum* (Söderholm *et al.*, 2011). Furthermore, CSPs were also shown to have roles under other stress conditions (Schmid *et al.*, 2009; Loepfe *et al.*, 2010). Interestingly, no homologues for CSP-encoding genes have been found in the sequenced genomes of psychrotrophic Group II *C. botulinum* type E strains (Söderholm *et al.*, 2013). In addition to CSPs, the so-called DEAD-box RNA helicases (Jarmoskaite & Russell, 2011) have been found to function in the destabilization of the cold-generated RNA secondary structures in several foodborne pathogens (Pandiani *et al.*, 2010; Markkula *et al.*, 2012b; Palonen *et al.*, 2012). Two DEAD-box helicases were shown to play additional roles in the tolerance to heat, alkali and oxidative stress in *Listeria monocytogenes* (Markkula *et al.*, 2012a) and in oxidative stress in *Clostridium perfringens* (Briolat & Reysset, 2002). No data on the role of DEAD-box helicases in *C. botulinum* exists to date.

An important aspect of cold-induced stress in the bacterium is the solidification of its lipid membrane. To counter this effect, the cell must increase the proportion of low melting-point fatty acids (FAs) such as unsaturated or *anteiso*-branched FAs in its cytoplasmic membrane (Suutari & Laakso, 1994). Two major strategies that accomplish this objective have been identified in bacteria. First, the existing membrane fatty acids (FA) can be desaturated (Suutari & Laakso, 1994). A cold-induced lipid desaturase

system (Des) has been identified in *B. subtilis* (Aguilar *et al.*, 1998), and it was found to be under direct transcriptional control of the temperature-responsive TCS, DesK/DesR (Aguilar *et al.*, 1999; Aguilar *et al.*, 2001; Cybulski *et al.*, 2002). However, this strategy is limited to aerobic bacteria, as the Des system is oxygen-dependent (Altabe *et al.*, 2003). Thus, considerably different approaches for rapid membrane fluidity adjustment are probably utilized by the strictly anaerobic clostridia. A second strategy for membrane function restoration is to increase the amount of lower melting point FAs in the membrane by *de novo* synthesis. This is accomplished by adjusting the FA synthesis mechanisms for increased production of unsaturated FAs, and by increasing the ratio of *anteiso*-to-*iso* branched FAs produced (Kaneda, 1991; Suutari & Laakso, 1994; Klein *et al.*, 1999; Zhu *et al.*, 2005; Singh *et al.*, 2009). Group II *C. botulinum* has been reported to adjust its membrane lipid composition according to temperature (Evans *et al.*, 1998), however, the mechanisms behind this phenomenon were not characterized.

Another mechanism implicated in bacterial tolerance to cold or hyperosmotic stress is the uptake and synthesis of cryoprotective small molecules. Compatible solutes such as glycine betaine, carnitine, choline and proline have been associated with cold and hyperosmotic tolerance in *B. subtilis* (Hoffmann & Bremer, 2011), and in *L. monocytogenes* (Ko *et al.*, 1994; Angelidis *et al.*, 2002). Furthermore, the disaccharide trehalose has been shown to possess cryo- and osmoprotective characteristics (Klein *et al.*, 1991; Elbein *et al.*, 2003; Duong *et al.*, 2006; Termont *et al.*, 2006). As far as the author is aware there exist no data on stress-protective functions of these molecules for *C. botulinum*.

Apart from SigB-mediated transcriptional control (Brigulla *et al.*, 2003; Hecker *et al.*, 2007; Chan *et al.*, 2008), the regulation of the cold stress response in bacteria has been attributed to several other transcriptional factors. For example, the alternative sigma factor, SigL, has been implicated in cold tolerance of *B. subtilis* (Wiegeshoff *et al.*, 2006) and also of *L. monocytogenes* (Raimann *et al.*, 2009; Mattila *et al.*, 2012). In addition to the DesK/DesR TCS described above, a multitude of TCSs have been shown to react to temperature change (Palonen *et al.*, 2011). In the plant pathogen *Pseudomonas syringae*, a modified TCS that consists of one sensor protein and two regulator proteins, has been shown to regulate virulence temperature-dependently (Ullrich *et al.*, 1995). The LisK/LisR, Lmo1173/Lmo1172, and Lmo1061/Lmo1060 TCSs were linked to cold shock response but were considered to be irrelevant for long-term cold growth by *L. monocytogenes* (Chan *et al.*, 2008). The primarily ferrous iron-responsive TCS FirRS of *Haemophilus influenzae* was induced by cold shock (Steele *et al.*, 2012) but the role of this TCS in cold tolerance was not characterized. In the foodborne pathogen *Yersinia pseudotuberculosis*, the majority of its TCS-encoding genes exhibited significantly higher expression levels at 3 °C than at 28 °C. Furthermore, the chemotaxis and motility-associated TCS CheA/CheY in *Y. pseudotuberculosis* was found to be important under cold

growth conditions (Palonen *et al.*, 2011). The genome of *C. botulinum* ATCC 3502 harbors 30 putatively TCS-encoding genes (Wörner *et al.*, 2006; Sebaihia *et al.*, 2007); of these, the CBO2307/CBO2306 TCS has been shown to be significantly induced upon cold shock and to be important in cold adaptation (Derman *et al.*, 2013a).

Responses to other foodborne stress factors

The sensing of heat stress is directly mediated by the increasing number of misfolded proteins (Mogk *et al.*, 1998). The response increases the number of chaperone proteins, i.e. proteins that aid the correct folding of other proteins (Hartl & Hayer-Hartl, 2002) to ensure correct folding of proteins. The class I heat shock chaperone genes *grpE*, *dnaK*, *dnaJ*, *groES*, and *groEL* were significantly induced upon heat shock in Group I *C. botulinum* ATCC 3502 (Selby *et al.*, 2011). Moreover, those authors also reported that DnaK was important in the tolerance to heat, hyperosmotic and low pH stresses. Upon heat shock, the growth of bacteria usually ceases in order for the cell to adapt, and then shortly afterwards the exponential growth is restored (Schulz & Schumann, 1996). Interestingly, no clear adaptation phase was noted for the heat shock response of Group I *C. botulinum*, though prolonged induction of the investigated HSPs was noted (Selby *et al.*, 2011).

To counter acidic stress, several general and specific stress responses are induced, including proton efflux pumps and molecular chaperones (Cotter & Hill, 2003; Mols & Abee, 2011b). Bacteria respond to low water activity-induced hyperosmotic stress by attempting to increase the amounts of intracellular solutes in order to maintain cell shape, turgor and membrane function (Kempf & Bremer, 1998; Krämer, 2010). The TCSs EnvZ/OmpR (Leonardo & Forst, 1996) and KdpD/KdpE (Walderhaug *et al.*, 1992) were shown to play a central role in bacterial osmosensing. The KdpD/KdpE TCS is thought to respond to both intra- and extracellular K^+ concentrations (Laermann *et al.*, 2013). The same TCS was demonstrated to possess a similar osmosensing function in *C. acetobutylicum* (Treuner-Lange *et al.*, 1997). Close homologues for the Kdp-encoding genes are found in sequenced Group II genomes; however, their functions have not been characterized as yet. No homologues for the Kdp-encoding genes can be found in the available Group I *C. botulinum* genomes.

The primary effects of several stress factors that bacteria encounter in food processing and storage, in addition to an overview of cellular responses elicited to counter these factors are summarized in Table 1.

2.2.3 Overlap of stress responses: cross-protection and mild stress-induced increased robustness

Significant overlap of the protective properties of several stress tolerance-related factors that are recruited against different environmental stressors has been observed (Höper *et al.*, 2005). An important observation to

Table 1 Food processing-induced environmental stress in bacteria

Stress	Primary effects on cell	Specific cellular responses	Reference
Cold	Hampered ribosome function. Inefficient enzyme function due to the deceleration of reaction kinetics. Impaired membrane function due to lipid solubilization.	Ribosome adaptation. Alterations in membrane fatty acid profile. Destabilization of nucleic acid secondary structures. Synthesis of cold-shock chaperones. Uptake and synthesis of cryoprotectants e.g. glycerine, trehalose and compatible solutes.	Wouters et al., 2000; Inouye & Phadtare, 2004; Phadtare, 2004
Heat	Denaturation of proteins. Damage to nucleic acids. Membrane damage.	Synthesis of heat-shock chaperones. Degradation of misfolded proteins.	Hartl & Hayer-Hartl, 2002; Schumann, 2003
Low pH	Destabilization of the membrane potential. Disturbed function of enzymes. Membrane and DNA damage	Proton efflux. Synthesis of chaperone proteins.	Cotter & Hill, 2003; Mols et al., 2010; Mols & Abee, 2011b; Desriac et al., 2013
Low water activity	Efflux of water from the cell. Loss of cell turgor and cell shape.	Uptake and synthesis of solutes to maintain cell shape and turgor.	Kempf & Bremer, 1998; Burg & Ferraris, 2008; Krämer, 2010
Ethanol	Increase in membrane permeability. Protein denaturation and disturbances of protein folding.	Heat-shock chaperone induction. Degradation of misfolded proteins.	Ingram, 1990; Seydlova et al., 2012

consider with regard to the cross-activation of stress responses is the identification of a secondary oxidative stress response when encountering specific primary stresses of various types (Höper *et al.*, 2005; Mols *et al.*, 2010; Mols & Abee, 2011a; Desriac *et al.*, 2013). Notably, the induction of genes related to oxidative stress was observed upon cold shock in *B. subtilis* (Kaan *et al.*, 2002), and also in cold growth in *L. monocytogenes* and in *Psychrobacter arcticus* (Liu *et al.*, 2002; Bergholz *et al.*, 2009). These observations suggest that secondary oxidative stress is an important component of cold stress. Moreover, several previously uncharacterized genes identified only as players in the "general" stress response have since been shown to confer specific resistance to oxidative stress, which suggests that the oxidative stress response plays an important role in countering a myriad of other stress conditions (Reder *et al.*, 2012a).

A cellular stress response elicited against a specific food-processing or storage-related stressor has been shown in several occasions to confer protection also against future stress of a different type (Browne & Dowds, 2001; Periago *et al.*, 2002; den Besten *et al.*, 2006; Mitchell *et al.*, 2009; den Besten *et al.*, 2010a; den Besten *et al.*, 2010b). A similar phenomenon was observed in disinfectant resistance of *L. monocytogenes*, where exposure to sub-lethal concentrations of desinfectants conferred increased resistance not only to the initial substance, but also to other desinfectans (Lundén *et al.*, 2003). Increased robustness against possible future treatments is of extreme importance in the hurdle-based design of food safety measures (Leistner, 2000).

Although the tolerance of *C. botulinum* against single or combined simultaneous effects of preservative measures is well studied (Graham *et al.*, 1996; Graham *et al.*, 1997; Lindström *et al.*, 2006; Peck, 2006), the effect of successive treatments on the development of robustness requires further attention. A differential induction of robustness that resulted from varying combinations of sublethal treatments has been documented in *L. monocytogenes* (Koutsoumanis *et al.*, 2003; Skandamis *et al.*, 2008). Importantly, the degree of induction of the cross-protective response has been shown to be at least partially temperature-dependent (Koutsoumanis *et al.*, 2003; den Besten *et al.*, 2010b; den Besten *et al.*, 2013).

2.3 Sporulation

A central means of survival for bacteria of the genera *Bacillus* and *Clostridium* under nutrient deprived-conditions and extreme environmental stress is the formation of endospores. The spore is considered to be an inert, dormant entity. Spores usually present considerably high resistance to extreme temperatures, desiccation, extreme pHs, salinity, UV irradiation, and importantly for the strictly anaerobic clostridia, oxygen (Nicholson *et al.*, 2000). The ubiquitous presence of heat-resistant spores of several

pathogenic bacteria, including *C. botulinum*, in the environment and subsequently in food raw materials, presents a central and major hazard in food processing (Lindström *et al.*, 2006).

2.3.1 The sporulation cascade and its regulation

Sporulation is a terminal choice for the cell: after a certain amount of progression of the sporulation cascade, the bacterium can no longer revert back to the vegetative state (Narula *et al.*, 2012). When the environmental and nutritional conditions again become favourable, the spore senses these signals and initiates germination and subsequently resumes vegetative growth (Setlow, 2003). The sporulation process consumes considerable amounts of the usually scarce energy, thus necessitating the strict control over the mechanisms responsible for initiating the sporulation cascade events. The model organism for spore-forming bacteria is *B. subtilis*, for which the events and regulatory networks of the sporulation cascade have been thoroughly characterized (Piggot & Hilbert, 2004).

The events of the sporulation cascade are divided into eight stages, 0-VII, according to morphological changes that occur in the cell (Waites *et al.*, 1970; Piggot & Hilbert, 2004). Stage 0 of the pathway is characterized by the elongation of the cell. A chromatin filament formation occurs at stage I though morphologically this stage closely resembles stage 0 (Waites *et al.*, 1970). During stage II, the cell divides asymmetrically into the mother cell and prespore. In stage III of sporulation, the mother cell engulfs the prespore. In stage IV of the pathway cortex formation around the engulfed prespore occurs. Then at stage V, the spore coat and germination proteins are added to the spore's cortex; these are necessary for the spores to germinate in response to specific signals (Henriques & Moran, 2007). During stage VI, the maturation of the spore takes place. Lastly, the mother cell lyses and releases the mature spore completing stage VII and the whole sporulation process.

Regulation of the sporulation cascade

In *Bacillus* and *Clostridium* genera, *spo0A* encodes the master switch of sporulation. Spo0A is activated by phosphorylation, which in *Bacillus* is achieved via a phosphorelay system (Burbulys *et al.*, 1991; Piggot & Hilbert, 2004). However, this system is not found in *Clostridium*: instead orphan kinases in *C. botulinum* (Wörner *et al.*, 2006) and in *C. acetobutylicum* (Steiner *et al.*, 2011) were proposed to phosphorylate Spo0A and thereby activate the sporulation cascade. The Spo0A regulation is dose-responsive. Spo0A-P possesses variable affinity to different promoters, which allows the precisely-timed expression for different parts of the Spo0A regulon (Fujita *et al.*, 2005).

In *B. subtilis,* the cascade downstream of Spo0A consists of subsequent activation of the mother cell- and prespore-specific sets of regulons that are coordinated by clockwork activation of a set of alternative sigma factors

(Hilbert & Piggot, 2004). Phosphorylated Spo0A (Spo0A-P) positively regulates *sigF*, the first of four downstream alternative sigma factors at stage 0 (Hilbert & Piggot, 2004). During stage I, the *spoIIAA* operon (containing *sigF* and *sigE*) is expressed before stage II of the sporulation pathway begins. After asymmetric division in stage II, SigF is activated in the prespore, while in the mother cell, pro-SigE is cleaved resulting in activated SigE. The activation of SigE is the point-of-no-return in the sporulation cascade of *B. subtilis* (Narula *et al.*, 2012). The activation of SigE subsequently activates SigG, which further regulates sporulation- and germination-related genes, including the one involved in the processing of pro-SigK. Activated SigK transcribes the stage V coat- and germination protein-encoding genes in the mother cell (Eichenberger *et al.*, 2004; Henriques & Moran, 2007).

Many sporulation factors that are characterized in *B. subtilis* are also encoded by clostridial genomes (Paredes *et al.*, 2005). Genes for all four sigma factors (*sigF*, *sigE*, *sigG*, and *sigK*) are present in the *C. botulinum* ATCC 3502 genome (Sebaihia *et al.*, 2007), and in the genomes of *C. acetobutylicum* (Jones *et al.*, 2008), *C. perfringens* (Harry *et al.*, 2009; Paredes-Sabja & Sarker, 2009), and in *C. difficile* (Fimlaid *et al.*, 2013).

Nonetheless, differences in the regulation of the sporulation pathways between clostridia and bacilli and also between different species of *Clostridium* have been reported (Paredes *et al.*, 2005; Jones *et al.*, 2008; Fimlaid *et al.*, 2013). The SigK regulator appears in the sporulation regulatory pathway across different species. In *C. perfringens*, sporulation was halted at an earlier stage in a *sigK* mutant than in a *sigE* mutant (Harry *et al.*, 2009), whereas in *B. subtilis sigK* mutants the cycle stops only at late-stage sporulation (Hilbert & Piggot, 2004). The difference between these two genera suggests that SigK in some clostridia may play a role in the early stages of sporulation, as opposed to the late-stage role in *B. subtilis*. Initially, Santangelo and colleagues (Santangelo *et al.*, 1998) suggested the sporulation sigma factors in *C. acetobutylicum* follow the expression pattern seen in *B. subtilis*. However, a more recent study demonstrated *sigK* expression at earlier stages of sporulation of *C. acetobutylicum*, before collapsing and peaking again in the later stages of sporulation (Jones *et al.*, 2008). These findings are similar to the biphasic *sigK* expression observed in *C. perfringens* (Harry *et al.*, 2009). Finally, SigK in *C. difficile* behaved similar to that in *B. subtilis*, i.e. SigK was strictly associated with the final stages of sporulation (Hilbert & Piggot, 2004; Fimlaid *et al.*, 2013; Pereira *et al.*, 2013; Saujet *et al.*, 2013).

2.3.2 Integration of the stress response into the decision-making network of sporulation

In *B. subtilis*, the decision-making networks of sporulation and stress adaptation were recently proposed to be woven together via a novel mechanism (Reder *et al.*, 2012b; Reder *et al.*, 2012c). As described above,

the initation of the sporulation cascade is triggered by increasing amounts of phosphorylated Spo0A (Spo0A-P). A major inhibiting factor of this phenomenon in *B. subtilis* is Spo0E, which exerts its action through direct dephosphorylation of Spo0A-P (Ohlsen *et al.*, 1994). The *spo0E* operon was reported to be transcribed by the general stress sigma factor SigB in *B. subtilis* (Reder *et al.*, 2012c). Furthermore, the induction of the SigB-dependent stress response by the exposure to ethanol resulted in a sporulation-deficient phenotype (Reder *et al.*, 2012b). It was confirmed that this phenotype resulted from SigB-dependent induction of the *spo0E* operon, resulting in decreased amounts of active Spo0A-P (Reder *et al.*, 2012b). The authors also suggest that the energy-expensive decision to sporulate can be considered as the "last resort" for the cell, whereas adaptation and survival in a "vegetative dormant" state is preferred for most situations (Reder *et al.*, 2012b; Reder *et al.*, 2012c). The negative feedback to the sporulation cascade exerted by the induction of the stress response supports this hypothesis.

Interconnections between the sporulation cascade and stress response in *C. botulinum* are unknown as yet. The lack of a homologue for *sigB* in the *C. botulinum* genome (Sebaihia *et al.*, 2007) suggests alternative mechanisms for stress response, and subsequently different approaches to those of bacilli in integrating the sporulation and stress response pathways may exist.

3. AIMS OF THE STUDY

1. Characterize the transcriptomic response of *Clostridium botulinum* ATCC 3502 to temperature downshift (I)

2. Investigate the role of the two-component system CBO0366/CBO0365 and its regulon in cold tolerance of *C. botulinum* ATCC 3502 (II-III)

3. Determine the role of the alternative sigma factor SigK in the sporulation (IV) and stress tolerance (V) of *C. botulinum* ATCC 3502

4. MATERIALS AND METHODS

4.1 Bacterial strains, plasmids and culture conditions

The sequenced (Sebaihia *et al.*, 2007) (Group I, toxin type A) *C. botulinum* type strain ATCC 3502 was used as a wild-type control strain, and as a parental strain for genetic modifications. Strains and plasmid vectors are presented in Table 2. *C. botulinum* strains were grown on tryptone-peptone-glucose-yeast extract (TPGY) (BD, Franklin Lakes, USA) plates or in TPGY (BD) broth, that was supplemented with antibiotics (Sigma Aldrich, St. Louis, USA) when appropriate. All *C. botulinum* cultivations were performed anaerobically, either in an anaerobic workstation with an internal atmosphere of 85% N_2, 10% CO_2 and 5% H_2 (MG-1000, Don Whitley Scientific, Shipley, UK) or in airtight jars with anaerobiosis that had been established by using oxygen-adsorbing sachets (Oxoid AnaeroGen, Thermo Fisher Scientific, Waltham, USA). *E. coli* strains were aerobically cultivated on Luria-Bertani (LB) (Sigma Aldrich) agar plates or in LB (Sigma Aldrich) broth, and were supplemented with antibiotics as necessary.

4.2 Growth experiments for gene expression analysis (I-V)

For RNA isolation for DNA microarray and RT-qPCR analysis of *C. botulinum* upon cold shock (I, III), and non-quantitative RT-PCR (III), the bacterial cultures were grown at 37 °C in TPGY medium until the culture reached an OD_{600} of 1.0. Then the cultures were subjected to rapid temperature downshift to 15 °C. Five-ml samples were taken from the cultures immediately before the temperature downshift, and after 1 h and 5 h of anaerobic incubation at 15 °C. For gene expression analysis upon cold shock and optimal growth (II, V), cultures were grown at 37 °C until they reached an OD_{600} of 1.0. Subsequently they were split into two halves, one half was subjected to a temperature downshift to 15 °C and the other half held at the temperature of 37 °C. One-ml samples were taken before splitting the cultures in half, and then immediately after the cold shock from the cold-shocked culture, and subsequently at 30 min, 2 h and 5 h of incubation at 15 °C. Samples from the cultures that were held at 37 °C were taken at the same time points as the cold incubation for comparison.

Cultures were grown at 37 °C for 14 h for the gene expression analysis of the sporulation cascade (IV). The samples were collected at 8, 10, 12, and 14 h after inoculation

Table 2 Bacterial strains and plasmid vectors

Strain or plasmid	Relevant properties	Source
Bacterial strains		
Clostridium botulinum		
ATCC 3502	Wild type control and parental strain	ATCC[1]
ATCC 3502 *cbo0097*::intron 124|125AS	Insertional disruption of *cbo0097* at base 124 in the antisense orientation, *erm*	I
ATCC 3502 *cbo0097*::intron 84|85S	Insertional disruption of *cbo0097* at base 84 in the sense orientation, *erm*	I
ATCC 3502 *cbo0477*::intron 152|153AS	Insertional disruption of *cbo0477* at base 152 in the antisense orientation, *erm*	I
ATCC 3502 *cbo0477*::intron 111|112S	Insertional disruption of *cbo0477* at base 111 in the sense orientation, *erm*	I
ATCC 3502 *cbo0558A*::intron 121|122AS	Insertional disruption of *cbo0558A* at base 121 in the antisense orientation, *erm*	I
ATCC 3502 *cbo0558A*::intron 114|115S	Insertional disruption of *cbo0558A* at base 114 in the sense orientation, *erm*	I
ATCC 3502 *cbo0365*::intron-erm	Insertional disruption of *cbo0365* at base 48 in the sense orientation, *erm*	II
ATCC 3502 *cbo0366*::intron-erm	Insertional disruption of *cbo0366* at base 267 in the sense orientation, *erm*	II
ATCC 3502/pMTL82153	ATCC 3502 parent strain with empty plasmid vector, *catP*	II
ATCC 3502 /pMTL82153::*cbo0366*	ATCC 3502 parent strain with an overexpression plasmid vector, *erm*, *catP*	II
ATCC 3502 *cbo0751*::intron 254|255AS	Insertional disruption of *cbo0751* at base 254 in the antisense orientation, *erm*	III
ATCC 3502 *cbo0753*::intron 196|197AS	Insertional disruption of *cbo0753* at base 196 in the antisense orientation, *erm*	III
ATCC 3502 *cbo1407*::intron 428|429AS	Insertional disruption of *cbo1407* at base 428 in the antisense orientation, *erm*	III
ATCC 3502 *cbo2525*::intron 122|123AS	Insertional disruption of *cbo2525* at base 122 in the antisense orientation, *erm*	III
ATCC 3502 *cbo2847*::intron 509|510AS	Insertional disruption of *cbo2847* at base 509 in the antisense orientation, *erm*	III
ATCC 3502 *cbo3199*::intron 467|468AS	Insertional disruption of *cbo3199* at base 467 in the antisense orientation, *erm*	III
ATCC 3502 *cbo3202*::intron 167|168AS	Insertional disruption of *cbo3202* at base 48 in the sense orientation, *erm*	III
ATCC 3502 *sigK*-296a	Insertional disruption of *cbo2541* at base 467 in the antisense orientation, *erm*	IV
ATCC 3502 *sigK*-427s	Insertional disruption of *cbo2541* at base 48 in the sense orientation, *erm*	IV
ATCC 3502/pMTL82151	ATCC 3502 parent strain with an empty plasmid vector, *catP*	IV

Table 2 Continued

Strain or plasmid	Relevant properties	Source
ATCC 3502 *sigK*-427s/pMTL82151	ATCC 3502 *sigK*-427s with an empty plasmid vector, *erm*, *catP*	IV
ATCC 3502 *sigK*-427s/pMTL82151::*sigK*	ATCC 3502 *sigK*-427s with a complementation plasmid vector, *erm*, *catP*	IV
ATCC 3502 *sigK*-296a/pMTL82151	ATCC 3502 *sigK*-296a with an empty plasmid vector, *erm*, *catP*	IV
ATCC 3502 *sigK*-296a/pMTL82151::*sigK*	ATCC 3502 *sigK*-296a with a complementation plasmid vector, *erm*, *catP*	IV
Escherichia coli		
CA434	Conjugation donor	University of Nottingham
TOP10	General purpose cloning strain, electrocompetent	Life Technologies
Rosetta 2(DE3)pLysS	Recombinant expression host, pRARE2 encoding seven rare tRNAs, *catP*	Novagen
Rosetta 2(DE3)pLysS::pET-28b(+)-*cbo0365*-HIS	Rosetta 2(DE3)pLysS harboring CBO0365-HIS protein overexpression vector	III
Plasmids		
pMTL007	Clostridial mutagenesis vector (ClosTron). L1.LtrB intron with *ermB* RAM, *catP*	University of Nottingham (Heap et al., 2007)
pMTL007C-E2	Clostridial mutagenesis vector (ClosTron). L1.LtrB intron with *ermB* RAM, constitutive intron expression under *fdx* promoter, *catP*	University of Nottingham (Heap et al., 2010)
pMTL82151	Modular clostridial shuttle vector, *catP*	University of Nottingham (Heap et al., 2009)
pMTL82153	Modular clostridial shuttle vector, constitutive *fdx* promoter, *catP*	University of Nottingham (Heap et al., 2009)
pMTL007C-E2::*cbo0097*::intron 124|125AS	pMTL007C-E2 targeting base 124 of *cbo0097* in the antisense orientation	I
pMTL007C-E2::*cbo0097*::intron 84|85S	pMTL007C-E2 targeting base 84 of *cbo0097* in the antisense orientation	I
pMTL007C-E2::*cbo0477*::intron 152|153AS	pMTL007C-E2 targeting base 152 of *cbo0477* in the sense orientation	I
pMTL007C-E2::*cbo0477*::intron 111|112S	pMTL007C-E2 targeting base 111 of *cbo0477* in the antisense orientation	I
pMTL007C-E2::*cbo0558A*::intron 121|122AS	pMTL007C-E2 targeting base 121 of *cbo0558A* in the sense orientation	I
pMTL007C-E2::*cbo0558A*::intron 114|115S	pMTL007C-E2 targeting base 114 of *cbo0558A* in the antisense orientation	I

Table 2 Continued

Strain or plasmid	Relevant properties	Source
pMTL007-*cbo0365*-48s	pMTL007 targeting base 48 of *cbo0365* in the sense orientation	II
pMTL007-*cbo0366*-267s	pMTL007 targeting base 267 of *cbo0366* in the sense orientation	II
pMTL82153-*cbo0366*	pMTL82153 harboring the CDS of *cbo0366* under the *fdx* promoter	II
PMTL007C-E2::*cbo0751*-254|255AS	PMTL007C-E2 targeting base 254 of *cbo0751* in the antisense orientation	III
PMTL007C-E2::*cbo0753*-196|197AS	PMTL007C-E2 targeting base 196 of *cbo0753* in the antisense orientation	III
PMTL007C-E2::*cbo1407*-428|429AS	PMTL007C-E2 targeting base 428 of *cbo1407* in the antisense orientation	III
PMTL007C-E2::*cbo2525*-122|123AS	PMTL007C-E2 targeting base 122 of *cbo2525* in the antisense orientation	III
PMTL007C-E2::*cbo2847*-509|510AS	PMTL007C-E2 targeting base 509 of *cbo2847* in the antisense orientation	III
PMTL007C-E2::*cbo3199*-467|468AS	PMTL007C-E2 targeting base 467 of *cbo3199* in the antisense orientation	III
PMTL007C-E2::*cbo3202*-167|168AS	PMTL007C-E2 targeting base 167 of *cbo3202* in the antisense orientation	III
pET-28b(+)	Recombinant protein expression vector, *kanR*	Novagen
pET-28b(+)-*cbo0365*-HIS	pET-28b(+) harboring the CDS of *cbo0365* with N-terminal 6-His tag	III
pMTL007C-E2::Cbo-*sigK*-296a	pMTL007C-E2 targeting base 296 of *cbo2541* in the antisense orientation	IV
pMTL007::Cbo-*sigK*-427s	pMTL007 targeting base 427 of *cbo2541* in the sense orientation	IV
pMTL82151::*sigK*	pMTL82153 harboring the CDS of *cbo2541* under its native promoter	IV

[1]ATCC, American Type Culture Collection

For the gene expression analysis of either hyperosmotic or acid shock (V), sterile NaCl was added to cultures grown at 37 °C to OD_{600} of 1.0, to a final concentration of 4.0% (w/v), or the pH of the cultures was lowered to 5.0 with HCl. The cultures were thereafter incubated at 37 °C for 5 h. Samples were withdrawn immediately before the relevant shock, and 1 min, 30 min, 1 h, 2 h, and 5 h thereafter.

All culture experiments for RNA isolations were performed as three independent biological replicates.

4.3 Transcriptional analysis

4.3.1 RNA isolation and reverse transcription (I-V)

Samples from conditions and time points described above were collected into sterile plastic tubes that contained ice-cold ethanol-phenol (9:1) stop solution (Sigma Aldrich) in a ratio of stop solution to sample of 1 to 5, then mixed thoroughly, and incubated on ice for 30 min. Cells were harvested by centrifugation at 8000 x G at 4 °C for 5 min. The pellets containing the cells were immediately frozen at -70 °C until RNA extraction.

RNA extraction was performed using the RNeasy Midi Kit (I, III) or RNeasy Mini Kit (II, IV-V) (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The cells were lysed with a solution containing 25 mg/ml lysozyme (Sigma Aldrich) and 250 IU/ml mutanolysin (Sigma Aldrich) in Tris-EDTA buffer (pH 8.0, Fluka BioChemica, Buchs, Switzerland). An additional DNase treatment was carried out using the DNA-free Kit (Ambion, Austin, USA) according to the manufacturer's instructions. The RNA yield and purity (A_{260}/A_{280}) were checked using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

Integrity of the RNA was confirmed by miniaturized gel electrophoresis in the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, USA).

For DNA microarray analysis (I, III), a total of 2 µg of each RNA sample was reverse-transcribed into cDNA and simultaneously labeled by fluorescent dyes. Each labeling reaction contained 0.2 µg/µl of random hexamers (Life Technologies, Carlsbad, USA), 0.01 M dithiothreitol (Life Technologies), 1.3 U/µl ribonuclease inhibitor (Life Technologies), 0.5 µM dATP, dTTP and dGTP, 0.2 µM dCTP, 1.7 nmol of Cy-3 or Cy-5 labeled dCTP (GE Healthcare, Pittsburgh, PA), 13 U/µl of SuperScript III reverse transcriptase (Life Technologies), and appropriate buffer (1 x First Strand Buffer, Life Technologies) and was subsequently incubated at 46 °C for 3 h. RNA hydrolysis and the reaction inactivation was performed by addition of 0.5 mM EDTA and 10 µl of 0.1 M NaOH and incubation at 70 °C for 15 min. The reactions were subsequently neutralized by the addition of 10 µl of 0.1 M HCl. The cDNA was purified by using the QIAquick PCR Purification Kit (Qiagen).

For RT-qPCR (I-V), 800 ng of each RNA sample was used for cDNA synthesis using the DyNAmo cDNA Synthesis Kit (Thermo Fisher Scientific) with random hexamer priming as instructed by the manufacturer. Two replicate RT reactions and a no-RT control reaction were run for each RNA sample.

For RT-PCR (III), 1 µg of *C. botulinum* ATCC 3502 wild-type RNA was reverse transcribed with a gene-specific primer using SuperScript III reverse transcriptase (Life Technologies) or 2 µl of water for a no-RT control reaction, as instructed by the manufacturer. The reactions were incubated at 55 °C for 60 min. RNA hydrolysis and reaction inactivation was performed as described above for the microarray cDNA synthesis.

4.3.2 Microarray analysis (I, III)

The *in situ* synthesized DNA microarrays (8x15K, Agilent Technologies) were custom-designed to cover 3641 chromosomal (out of the total of 3648) and all 19 plasmid-borne ORFs in the ATCC 3502 genome (Sebaihia *et al.*, 2007).

For array hybridizations biological replicate samples were labeled with either Cy5 or Cy3 as described above. A total of 300 ng of Cy3-labelled cDNA and 300 ng of Cy5-labelled cDNA were mixed into a final volume of 18 µl, and 0.1 mg/ml of salmon sperm DNA (Life Technologies) was added. The DNA was denatured at 95 °C for 2 min, then chilled on ice, and finally mixed with blocking agent (Hi-RPM GE Hybridization Kit, Agilent Technologies) and hybridization buffer as instructed by the manufacturer (Hi-RPM GE Hybridization Kit). A volume of 50 µl of the mix was pipetted onto the DNA microarrays. The arrays were hybridized at 65 °C overnight, and washed as instructed (Gene Expression Wash Buffer Kit, Agilent Technologies).

The slides were scanned at 532 and 635 nm using a 5-µm resolution with the Axon GenePix Autoloader 4200 AL (Molecular Devices, Sunnyvale, USA). Image processing was accomplished by using GenePix Pro 6.0 software (Molecular Devices) and data analysis with the *R limma* package (Smyth, 2005). Foreground and local background intensities of each spot were characterized by the mean and median pixel values of the respective spot. The local background signal was subtracted from the foreground signal using the "normexp" method with an offset value of 50 (Ritchie *et al.*, 2007). For comparison within arrays, signal intensities measured in the Cy5 and Cy3 channels were converted into a logarithmic ($\log_2$) scale and normalized using the *loess* method (Smyth & Speed, 2003). For comparison between arrays, quantile normalization was performed (Smyth & Speed, 2003). A moderated t-test with empirical Bayes variance shrinkage was applied to each probe on the array and the resulting *p*-values were converted into a false discovery rate (FDR) values (Smyth, 2005). A specific probe with a median unmodified *p*-value for the expression difference was chosen to represent each ORF. Of these, ORFs with FDR <0.05 were subsequently considered to have a significant difference in expression. Furthermore, of these significantly

differently expressed ORFs, those that had a $\log_2$ fold change of <-2.0 or >2.0 were included in the final expression analysis.

4.3.3 Quantitative real-time reverse transcription-PCR (RT-qPCR) (I-V)

Quantitative real-time reverse transcription-PCR (RT-qPCR) was performed to validate the expression values obtained from the microarray experiments (I, III), and to determine the expression of *cbo0366* and *cbo0365* upon optimal growth and upon cold stress (II). The expression of *sigK* (*cbo2541*) under optimal growth, and upon cold, hyperosmotic and low pH stress conditions (IV-V) was similarly determined by RT-qPCR. The DyNAmo Flash SYBR Green qPCR Kit (Thermo Fisher Scientific) was used according to the manufacturer's instructions to set up the RT-qPCR reactions. Each reaction consisted of 1X DyNAmo Flash SYBR Green Master Mix (Thermo Fisher Scientific), 0.5 µM of forward and reverse primer, and 4 µl of cDNA in a total volume of 20 µl. The reactions were performed in a Rotor-Gene RG3000 thermal cycler (Qiagen). The amplification efficiencies and the appropriate sample dilution factors for each primer pair were determined with a dilution series of pooled cDNA. The Rotor-Gene 6 software (Qiagen) was used to set the threshold fluorescence levels for each primer pair, and to calculate the reaction efficiencies. The reaction efficiencies of the primers varied between 88% and 105%. No-template controls were included in each run. A melt curve analysis was performed to confirm primer specificity.

The relative quantification of target gene expression, normalized to a reference gene (16S *rrn*), were determined by calculating the expression ratios of the target genes with the Pfaffl method (Pfaffl, 2001). This relative quantification incorporates the reaction efficiency of each primer pair into the ratio calculations. The Cq values for 16S *rrn* remained stable throughout the experiment, which underpinned its use as a reliable normalization control.

4.3.4 Non-quantitative reverse transcription PCR (RT-PCR) (III)

Reverse transcription PCR was performed on cDNA synthesized from wild-type ATCC 3502 RNA to investigate the transcript structure of the *cbo0364-cbo0366* locus. As a template, 1 µl of first-strand cDNA template, no-RT control, water, or ATCC 3502 genomic DNA was used. The reactions contained 200 µM dNTP mixture (Thermo Fisher Scientific), 2 U of DyNAzyme II DNA polymerase (Thermo Fisher Scientific), and 0.5 µM of each forward and reverse primer in 1X reaction buffer (Thermo Fisher Scientific).The primers were targeted at the intergenic or coding regions of the genes of the putative transcript.

4.4 Genetic manipulation of *C. botulinum*

4.4.1 Construction of mutants (I-IV)

ClosTron technology (Heap *et al.*, 2007; Heap *et al.*, 2010) was utilized for the construction of insertional knockout mutant strains of *C. botulinum* ATCC 3502. The technology is based on a targeted site-specific integration of a mobile group II intron into the genome that results in the disruption of the reading frame and subsequently in the inactivation of the desired gene. In publication II, and partly in publication IV, the intron targeting regions were mutated by SOE PCR to target desired genes. The mutated targeting regions were cloned into the pMTL007 vector, and subsequently transformed via conjugation from *E. coli* CA434 to the parental *C. botulinum* strain, and finally the intron expression was induced by IPTG. In studies I, III and partially IV, *de novo* synthesized intron targeting regions that had been cloned into pMTL007C-E2 vector were purchased (DNA2.0, Menlo Park, USA), and similarly transformed into the recipient wild-type cells. The pMTL007C-E2 vector expresses the intron constitutively (Heap *et al.*, 2010). Thus IPTG induction was omitted. Correct intron insertion sites and orientations were confirmed by PCR. A single intron insertion was confirmed by Southern blotting with a probe targeted to the inserted sequence. The intron insertion sites and orientations in the mutant strains, and the plasmid constructs, are presented in Table 2.

4.4.2 Complementation and overexpression (II, IV)

To complement the disruption of *sigK* a 1268-bp fragment that contained the CDS of *sigK*, 425 bp upstream (considered to harbor the natural promoter of *sigK*), and 138 bp downstream of the CDS, was PCR-amplified with a high-fidelity DNA polymerase (Phusion DNA Polymerase, Thermo Fisher Scientific) and cloned into pMTL82151 to make pMTL82151::*sigK*. The construct was verified by sequencing, and conjugated into the two *sigK* mutants from *E. coli* CA434.

To overexpress *cboo366* in the ATCC 3502 wild type *in trans*, the high-fidelity PCR amplified (Phusion DNA Polymerase, Thermo Fisher Scientific) CDS of *cboo366* was cloned into pMTL82153 under the *fdx* promoter to make pMTL82153-*cboo366*. The construct was then verified by sequencing. The overexpression vector and the empty pMTL82153 were transformed into the ATCC 3502 parent strain by conjugation.

4.5 Characterization of genetically modified *C. botulinum*

4.5.1 Growth curve analysis (I-III, V)

Growth experiments were conducted to compare the growth of the ATCC 3502 wild type, mutant, and overexpression strains under optimal and stress conditions. These experiments were done either in an automatic turbidity reader (Bioscreen C Microbiology Reader, Growth Curves, Helsinki, Finland) (II-III), or in culture bottles (I, V). Growth under optimal conditions (I-III, V) was investigated by incubating the strains at 37 °C in TPGY broth for 12 h. In the cold growth experiments, the strains were cultivated in TPGY broth at 15 °C for 14 d or at 20 °C for 7 d (II), or at 17 °C for 7 d (I, III, V). For the acid and hyperosmotic stress growth experiments (V), the TPGY broth was adjusted to pH 6.0, pH 5.3 or pH 5.0 by HCl, whereas 4.5% (w/v) NaCl was added to make the broth hyperosmotic. The strains were cultivated at 37 °C for 30 h in acidified TPGY, or for 60 h in 4.5% NaCl-supplemented TPGY. For growth under arsenic stress (III), TPGY supplemented by 0.1 % (v/v) sodium arsenite was used, and the strains were cultivated at 37 °C for 50 h or at 20 °C for 10 d. Three to five biological replicate cultures were grown in each experiment. The OD_{600} of the cultures was monitored at regular intervals either automatically (II-III) or manually without exposing the cultures to air (I, V). Growth curves were constructed by plotting the OD_{600} of each culture against time.

4.5.2 Global gene expression analysis (III)

Microarray analysis of the differences in global gene expression patterns between the wild type and the *cbo0365* mutant in mid-logarithmic growth and at 1 h after temperature downshift to 15 °C were carried out to identify the direct and indirect putative regulatory targets of the CBO0365 response regulator.

4.5.3 Sporulation (IV)

The *C. botulinum* ATCC 3502 parent strain, the *sigK* mutants, and the *in trans*-complemented *sigK* mutant strains were incubated at 37°C under anaerobic conditions for 7 days in order to determine their sporulation characteristics. Two samples of each culture were taken, one of which was heated at 80°C for 20 min to kill any vegetative cells, and the other left untreated. The heated and nonheated samples were serially diluted (10^{-1} to 10^{-5}) in TPGY broth which was supplemented with thiamphenicol when appropriate, and incubated at 37 °C for 48 h. Viable heat-resistant spores

were considered to be present when growth was observed after the incubation period in the heat-treated samples.

The presence of spores was visually confirmed by culturing colonies of the parent and both *sigK* mutant strains on TPGY agar plates for 7 d and then staining the samples on a glass slide with malachite green and safranin (Reynolds *et al.*, 2009).

4.5.4 Expression of the sporulation cascade genes *spo0A* and *sigF* (IV)

The relative transcription levels of *spo0A* and *sigF* between the parent and *sigK*-427s mutant strains were investigated at late-exponential (8 h of growth) and stationary (10 to 14 h of growth) phases with RT-qPCR.

4.6 Protein-DNA interaction experiments (III)

4.6.1 Cloning, overexpression and purification of recombinant CBO0365 protein

A high-fidelity PCR (Phusion DNA Polymerase, Thermo Fisher Scientific) amplified DNA fragment that contained the coding sequence of *cbo0365* was cloned into a pET-28b(+) expression vector (Thermo Fisher Scientific), thereby incorporating an N-terminal 6-His tag into the CDS. The plasmid constructs were verified by sequencing. Recombinant 6-His-tagged CBO0365 was overexpressed in *E. coli* Rosetta 2(DE3)pLysS (Thermo Fisher Scientific) and affinity purified with His-Bind Ni^{2+}-NTA resin (Thermo Fisher Scientific) according to the manufacturer's instructions. The purity of the protein was assessed by SDS-PAGE, and its concentration was approximated by the Bradford assay using BSA as a standard. The purified protein was dialyzed into storage buffer (50 mM HEPES [pH 7.5, Sigma Aldrich], 100 mM NaCl, 25 mM MgCl, 1 mM EDTA, 10% glycerol) with Novagen D-tube dialyzers (molecular weight cut-off 6-8 kDa) and stored at 4 °C for a maximum of two weeks.

4.6.2 Electrophoretic mobility shift assays

The ability of recombinant CBO0365 protein to bind *in vitro* to putative promoter sequences of the operons of interest was investigated by using nonradioactive electrophoretic mobility shift assays (EMSAs). We produced 5´-biotin labeled DNA probes for EMSA by PCR-amplifying DNA fragments that contained the putative promoter sequences of interest with Phusion DNA Polymerase (Thermo Fisher Scientific) using 5´-biotin labeled forward primers and unlabeled reverse primers. Cold probes were similarly produced

using only unlabeled primers. Additionally, labeled and cold negative control fragments derived from the coding sequence of 16S *rrn* were similarly constructed. The labeled probes were extracted from polyacrylamide gel and precipitated by ethanol, whereas the unlabeled probes were PCR purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific) to ensure a sufficient yield. The concentration and purity of the probes were determined by Nanodrop.

Prior to the DNA-protein binding reactions, 0 to 4 μM of 6-His-tagged CBO0365 protein was phosphorylated in binding buffer that contained 20 mM HEPES (pH 7.5, Sigma Aldrich), 60 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (Life Technologies), 0.3 mg/ml BSA, 5% glycerol and 50 mM lithium potassium acetyl phosphate (Sigma Aldrich) for 1 h at 25 °C. We tested the binding capacity of CBO0365 to the promoter fragments produced above, by adding 20 fmol of a 5´-biotin labeled DNA probe to the reactions and allowing binding to proceed for 30 min at 25 °C. The reactions were loaded into a pre-cast 5% native polyacrylamide gel (Bio-Rad, Hercules, USA) and run at 115 V for 90 to 160 min in pre-chilled 0.5X TBE buffer at 4 °C. The DNA fragments were transferred to a positively charged nylon membrane (Roche Applied Science, Indianapolis, USA), and membrane detection was performed by using a Pierce Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher Scientific) according to the manufacturer's instructions. Control reactions without acetyl phosphate were similarly performed, and additional reactions to determine the specificity of each DNA-protein interaction were performed with a 200-fold molar excess of unlabeled DNA probe added to the reactions with 4 μM protein.

5. RESULTS

5.1 Genomewide transcriptional analysis of *C. botulinum* upon cold shock (I)

Transcriptomic analysis of the *C. botulinum* ATCC 3502 cold-shock response revealed a set of 27 genes with significantly affected expression already 1 h after temperature downshift (Table 3). At 5 h after cold shock, an extensive metabolic remodeling was observed (Fig. 1). Altogether 409 differentially regulated genes were identified at this time point (Fig. 1). Notably, the induction of genes putatively related to adaptation, fatty acid biosynthesis, and regulation was observed. Several genes that encode for proteins with putative functions in oxidative stress response, cellular redox balance, and iron homeostasis were also significantly induced. Moreover, several genes that were annotated as DNA-binding regulatory proteins without further functional prediction or association to cold shock response in bacteria, were identified as significantly induced. Disruption of two of these putative regulators (*cbo0477* and *cbo0558A*) resulted in a deterioration of cold tolerance of *C. botulinum* ATCC 3502.

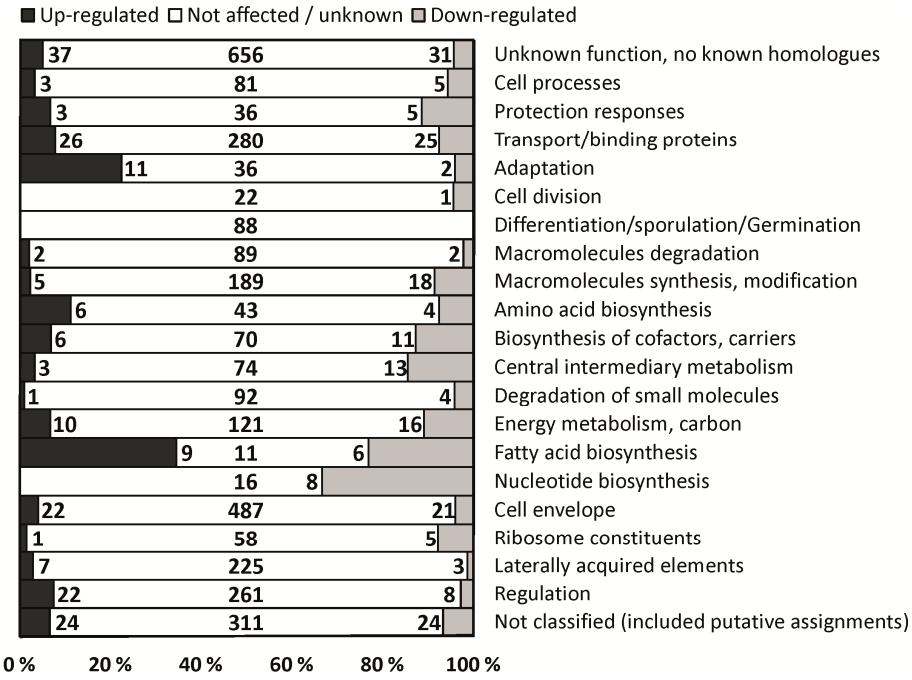

Figure 1 Distribution of significantly up- or down-regulated genes among functional categories 5 h after temperature downshift from 37 °C to 15 °C in *C. botulinum* ATCC 3502 (I).

5.2 The role of the two-component system (TCS) CBO0366/CBO0365 in cold tolerance of *C. botulinum* (I-III)

5.2.1 Expression and effect of disruption of the *cbo0366* and *cbo0365* genes on cold tolerance (I-III)

The CBO0366/CBO0365 TCS was found to be the most strongly-induced TCS upon cold shock as determined in the microarray analysis of the cold shock response of *C. botulinum* ATCC 3502 (I). Detailed RT-qPCR expression analysis confirmed the up-regulation of the TCS-encoding genes *cbo0365* and *cbo0366* upon cold shock, without this induction occurring under optimal growth (II). Moreover, down-regulation of the TCS genes was observed upon entry into the stationary phase (II). Inactivation of either of the TCS components resulted in deteriorated growth at 15 °C (Fig. 2A) and at 20 °C, but did not affect the phenotype at 37 °C (II). Over-expression of *cbo0366* in the ATCC 3502 wild-type strain improved growth at 15 °C, compared to the vector control (Fig. 2B) (II). Furthermore, inactivation of *cbo0365* resulted in impaired tolerance to sodium arsenite at 37 °C (Fig. 2E); the combined effect of 0.1 mM of sodium arsenite and a temperature of 20 °C completely abolished growth of the mutant (Fig. 2F) (III).

5.2.2 Identification of genes under direct and indirect transcriptional control of the CBO0365 response regulator (III)

We identified the CDSs under the putative regulation of the CBO0365 response regulator. This was done by comparing the transcriptomes of the wild-type ATCC 3502 and *cbo0365* mutant strains during growth at 37 °C and 1 h after a temperature downshift to 15 °C using DNA microarrays, which were based on the ATCC 3502 genome. The genes that were expressed significantly less or more (median $\log_2$ ratios <-2.0 or >2.0, false discovery rate [FDR] <0.05) in the *cbo0365* mutant, than in the wild-type strain were considered to be directly or indirectly positively or negatively regulated by CBO0365.

At 37 °C, a total of 150 chromosomal CDSs showed significantly different expression between the *cbo0365* mutant and the wild-type strain. The number of differentially expressed chromosomal CDSs at one hour after temperature downshift to 15 °C was 141. Moreover, all chromosomal CDSs differently expressed between the wild type and *cbo0365* mutant only at either of the two experimental temperatures were similarly less or more expressed at the other (FDR <0.05), albeit with a $\log_2$ expression difference falling outside the defined cut-off values. The chromosomal CDSs that were expressed less in the *cbo0365* mutant than in the wild type at both 37°C and 15°C were arranged in 6 loci.

Table 3 The genes repressed or induced <-2.0 or >2.0 log_2-fold 1 h after temperature downshift from 37 °C to 15 °C in *C. botulinum* ATCC 3502 (I).

Locus tag	Main class	Subclass	Product	Gene symbol(s)	log_2 fold change	
					1 h	5 h
CBO0038	Unknown function	Conserved in other than *E.coli*	hypothetical protein	*cbo0038*	-2.3	-2.6
CBO0791	Amino acid biosynthesis	Lysine	dihydrodipicolinate reductase	*dapB, cbo0791*	-2.0	-2.9
CBO2072	Central intermediary metabolism	Nucleotide interconversions	cytosine deaminase	*codA, cbo2072*	-2.7	-4.5
CBO2073	Transport/binding proteins	Other	cytosine permease	*codB, cbo2073*	-2.4	-2.6
CBO2459	Not classified	Not classified	phosphotransacetylase	*eutD, cbo2459*	-2.4	-1.3
CBO2539	Cell processes	Chemotaxis and mobility	twitching motility protein	*cbo2539*	-2.1	-3.0
CBO3056	Not classified	Not classified	coproporphyrinogen III oxidase	*cbo3056*	-2.5	-3.5
CBO3073	Unknown function	Conserved in other than *E.coli*	hypothetical protein	*cbo3073*	-2.5	-2.5
CBO3241	Nucleotide biosynthesis	Pyrimidine ribonucleotide biosynthesis	aspartate carbamoyltransferase	*pyrB, CBO3241*	-2.1	-4.6
CBO3444	Transport/binding proteins	Cations	Na/Pi-cotransporter family protein/PhoU family protein	*cbo3444*	-2.1	-2.3
CBO3544	Nucleotide biosynthesis	Purine ribonucleotide biosynthesis	ribose-phosphate pyrophosphokinase	*prsA, prs, cbo3544*	-2.7	-2.1
CBO0117	Not classified	Not classified	LysM domain-containing protein	*cbo0117*	2.2	3.7
CBO0282	Adaptation	Adaptations, atypical conditions	cold shock protein	*cspA, cbo0282*	2.7	3.9
CBO0389	Transport/binding proteins	Amino acids and amines	amino acid permease	*cbo0389*	4.0	7.5
CBO0558A	Regulation	GntR	GntR family transcriptional regulator	*cbo0558A*	3.1	5.1
CBO0559	Transport/binding proteins	Transport/binding proteins	ABC transporter ATP-binding protein	*cbo0559*	3.0	4.6
CBO0560	Cell envelope	G+ membrane	ABC transporter permease	*cbo0560*	2.7	4.9
CBO1106	Cell envelope	G+ membrane	hypothetical protein	*cbo1106*	2.2	2.6
CBO1332	Energy metabolism, carbon	Anaerobic respiration	nitrate reductase, iron-sulfur subunit	*cbo1332*	2.1	2.4
CBO1333	Energy metabolism, carbon	Anaerobic respiration	nitrate reductase, NADH oxidase subunit	*cbo1333*	2.2	3.3
CBO1386	Unknown function	Conserved in other than *E.coli*	hypothetical protein	*cbo1386*	2.7	4.7

Table 3 Continued

Locus tag	Main class	Subclass	Product	Gene symbol(s)	log_2 fold change	
					1 h	5 h
CBO1589	Protection responses	Cell killing	hemolysin	*cbo1589*	2.1	6.5
CBO1636	Adaptation	Osmotic adaptation	glycine betaine/L-proline ABC transporter ATP-binding protein	*opuCA, opuBA, proV, cbo1636*	2.7	5.3
CBO1637	Adaptation	Osmotic adaptation	glycine betaine/L-proline ABC transporter permease/substrate-binding protein	*opuCB, opuBB, cbo1637*	2.3	4.8
CBO2591	Unknown function	Unknown function	seryl-tRNA synthetase	*cbo2591*	2.8	7.6
CBO2592	Fatty acid biosynthesis	Fatty acid biosynthesis	hypothetical protein	*cbo2592*	3.4	8.1
CBO2802	Macromolecule synthesis, modification	RNA synthesis, modification, DNA transcription	ATP-dependent RNA helicase, DEAD/DEAH box family	*deaD, csdA, mssB, cbo2802*	2.1	3.1

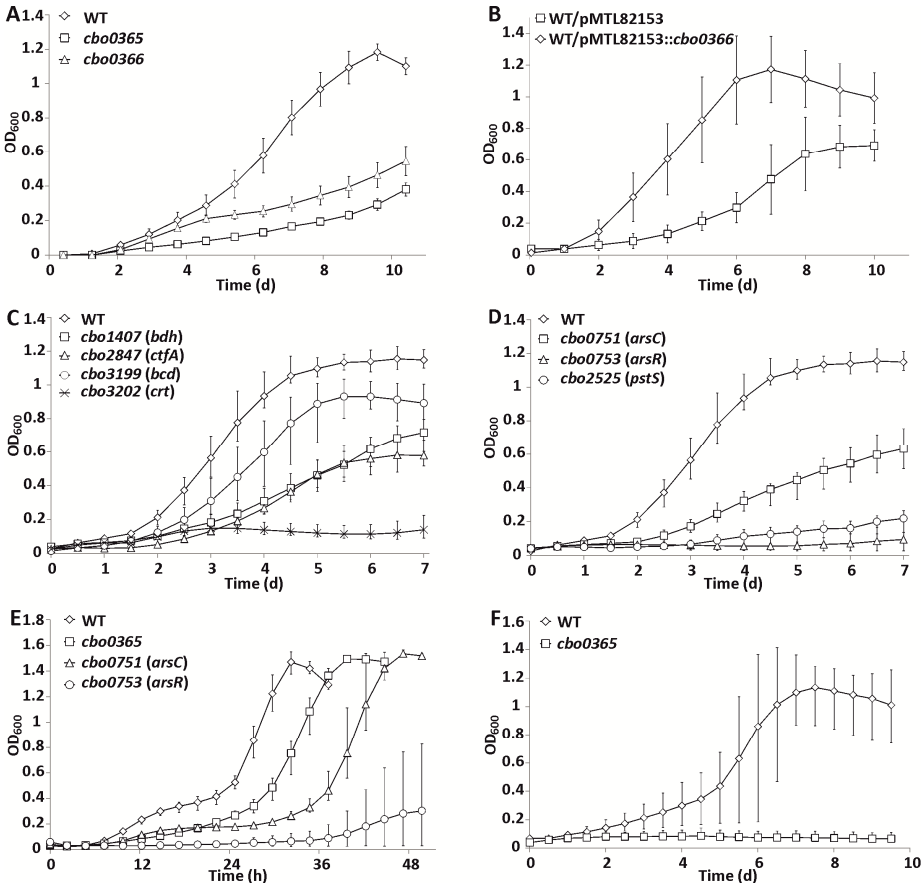

Figure 2 The role of the CBO0366/CBO0365 TCS, and genes of CBO0365-regulated metabolic pathways on cold and arsenite tolerance of *C. botulinum* ATCC 3502. **(A):** growth of ATCC 3502 wild type (WT), *cbo0365* and *cbo0366* mutants at 15 °C. **(B):** growth of ATCC 3502 wild type harboring an empty overexpression vector backbone, and ATCC 3502 constitutively overexpressing *cbo0366* at 15 °C. **(C):** growth of ATCC 3502 wild type (WT) and mutants of ABE fermentation-related *cbo1407*, *cbo2847*, *cbo3199*, and *cbo3202* at 17 °C. **(D)**: growth of ATCC 3502 wild type (WT) and mutants of arsenical resistance-related *cbo0751* and *cbo0753*, and phosphate uptake-related *cbo2525* at 17 °C. **(E):** growth of ATCC 3502 wild type (WT), *cbo0365*, *cbo0751*, and *cbo0753* mutants in presence of 0.1 mM sodium arsenite at 37 °C. **(F):** The growth of ATCC 3502 wild type (WT), and *cbo0365* mutant in presence of 0.1 mM sodium arsenite at 20 °C. Figure adapted from (II and III).

They also included the following; *cbo0043* that encodes for a putative RNA polymerase sigma factor, *cbo0344* that encodes for a metallopeptidase, *cbo3197-cbo3199* and *cbo3200-cbo3202* that were both related to acetone-butanol-ethanol (ABE) fermentation, 16 CDSs related to prophage 1 (entire prophage is *cbo1679-cbo1755*), and 49 CDSs related to prophage 2 (entire prophage is *cbo2313-cbo2394*). The chromosomal CDSs that were significantly expressed more highly in the *cbo0365* mutant than in the wild

type at 37°C and 15°C were arranged in 32 loci. These CDSs included those with predicted functions in sporulation (*cbo0069*),multidrug resistance (*cbo0072*), pantothenate biosynthesis (*cbo0422-cbo0425*), fatty acid biosynthesis (*cbo0502*), arsenical resistance (*cbo0753-cbo0756*), glucoside uptake (*cbo0880-cbo0883*), flagellar rotation (*cbo2226* and *cbo2227*), phosphate binding and transport (*cbo2521-cbo2525*), dehydrogenation (*cbo2850-cbo2852*), and a putative genomic island (*cbo3016-cbo3044*).

RT-PCR analysis confirmed the tricistronic transcription of *cbo0364-cbo0366*. *In vitro* phosphorylated CBO0365 was shown to bind to a 150-bp fragment that was derived from the non-coding DNA region that is located directly upstream of *cbo0364*: a finding that suggested CBO0365 directly controls the transcription of its own operon (Fig. 3A). Furthermore, binding to the promoter regions of *cbo3202* (Fig. 3B), *cbo2525* (Fig. 3C) and to the full-length non-coding region between *cbo0752* and *cbo0753* (Fig. 3D) was observed. No binding was observed to the 180-bp fragment derived from the sequence directly upstream of *cbo0753* (Fig. 3E) or to the negative control fragment (Fig. 3F). *In vitro* phosphorylation of CBO0365 for 60 min by acetyl phosphate was essential for any DNA-binding activity.

5.2.3 Effects of disruption of CBO0365-regulated metabolic pathways on cold tolerance (III)

We investigated the role of CBO0365-regulated metabolic pathways in the cold tolerance of *C. botulinum* ATCC 3502 by determining the growth characteristics of mutants of *cbo0751*, *cbo0753*, *cbo1407*, *cbo2847*, *cbo3199*, and *cbo3202* at 17 °C. Inactivation of *cbo3202* resulted in a complete inability to grow at 17 °C (Fig. 2C). A slight decrease in the growth rate of the *cbo3199* mutant at 17 °C was also observed (Fig. 2C). The *cbo1407* and *cbo2847* mutants both had a markedly lower growth rate at 17 °C than the wild-type strain (Fig. 2C). Markedly impaired growth was observed for the *cbo0751* mutant at 17 °C, and inactivation of *cbo0753* completely abolished growth at this temperature (Fig. 2D). This finding suggested an important role for these arsenic resistance protein-encoding genes in the cold tolerance of *C. botulinum* ATCC 3502. Similarly, inactivation of *cbo2525* resulted in a phenotype that was almost completely unable to grow at 17 °C (Fig. 2D). No differences in growth at 37 °C were observed between the wild-type strain and any of the mutant strains investigated. Furthermore, the requirement for intact arsenic resistance machinery in exerting arsenic tolerance of *C. botulinum* was demonstrated by the impaired growth of *cbo0751* and *cbo0753* mutants in the presence of 0.1 mM sodium arsenite (Fig. 2E).

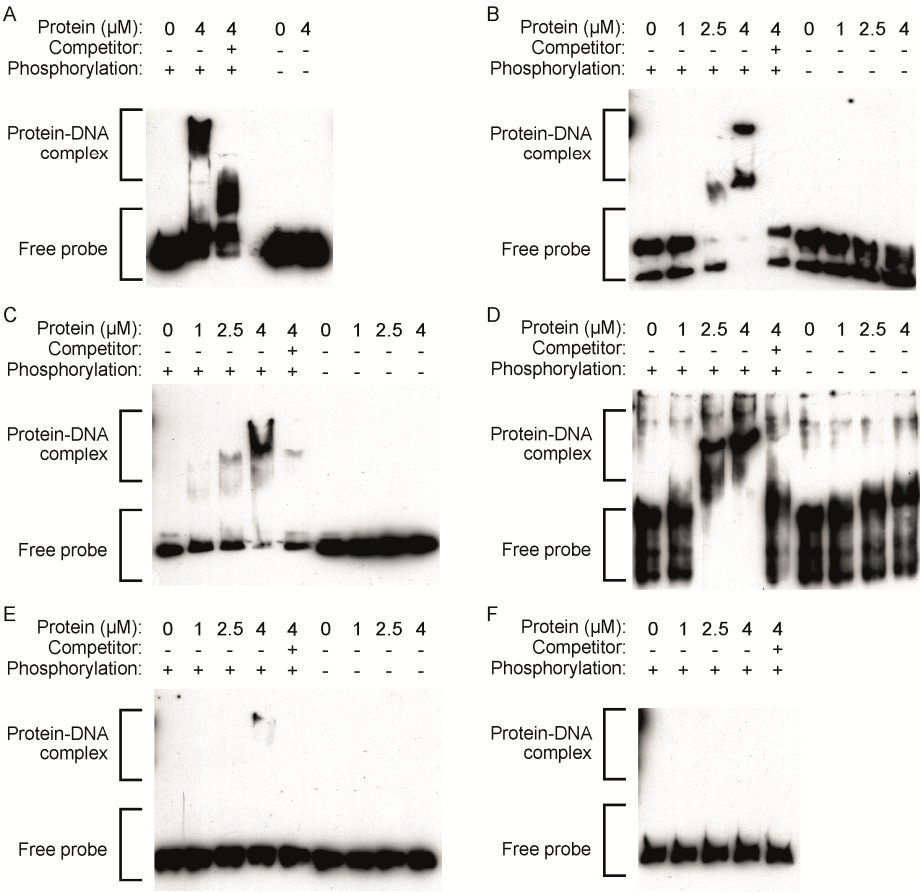

Figure 3 Electrophoretic mobility shift assay with phosphorylated CBO0365 protein showing the binding to double-stranded 5´-biotin-labeled DNA probes of putative promoter regions of *cbo0364* **(A)**: *cbo3202* (**B**): *cbo0753* **(C):** and *cbo2525* **(D**). No binding was observed to a short fragment directly upstream of *cbo0753* **(E)**, nor to a negative control fragment **(F)**. No DNA binding activity was observed for non-phosphorylated CBO0365 **(A-E)**. Specificity of the protein-DNA interactions between CBO0365 and the promoter fragments were confirmed by prevention of signal shifts in the presence of a 200-fold molar excess of unlabeled competitor probe. Furthermore, no binding to a negative control fragment derived from the coding sequence of 16S *rrn* was observed **(F)** (III).

5.3 The role of the alternative sigma factor SigK in sporulation and stress tolerance of *C. botulinum* (IV-V)

5.3.1 Disruption of *sigK* blocks sporulation (IV)

Both of the *sigK* mutants failed to show any growth after heat-treatment, which indicated a lack of sporulation, germination, or both, in response to a disruption of *sigK*. The complemented *sigK*-427s that contained pMTL82151::Cbo-*sigK* and the wild-type control that contained pMTL82151

alone, did show growth after heating, which demonstrated successful sporulation and germination. The complemented *sigK*-296a also demonstrated growth after heating, although the number of viable spores was 2 log units less than that for the parent strain. The unheated controls of each strain demonstrated growth at all dilutions. Furthermore, no visible spores were observed in malachite green and safranine stains of the *sigK*-427s and *sigK*-296a mutant strains. Additionally, elongated morphology was observed for the mutant cells, and in particular for the *sigK*-296a mutant.

5.3.2 Expression levels of *sigK* decrease upon entry into the stationary growth phase

The relative *sigK* transcription levels in the wild-type strain were 6.5- and 7.8-fold higher ($P<0.05$) at the late exponential phase (8 h post-inoculation) than those of the stationary phase samples (12 and 14 h post-inoculation, respectively). A large variation was observed among the biological replicates at 10 h, which corresponded to the entry into the stationary phase.

5.3.3 Disruption of *sigK* results in the decrease of relative expression of the sporulation regulators *spo0A* and *sigF* (IV)

Significantly higher levels of *spo0A* transcription were observed for the parent strain than for the *sigK*-427s mutant at 8 h (4.9-fold) and 10 h (2.3-fold) post-inoculation. A notable drop in transcription levels was observed in both the parent (28-fold) and *sigK*-427s (16-fold) strains at between 10 and 12 h. No difference between the parent and mutant *spo0A* transcript levels was observed at 12 h, whereas at 14 h after inoculation, a slightly higher (1.9-fold) expression was observed for the wild-type strain.

Similar to that found for *spo0A*, the relative *sigF* transcription levels were significantly higher (5.3-fold) in the parent than in the *sigK*-427s mutant strain at 8 h after inoculation. A non-significant trend in *sigF* transcription levels similar to that found for *spo0A* was observed at 10 h. At between 10 and 12 h, notable drops in the relative *sigF* transcription levels in both the parent strain (9.2-fold) and the mutant *sigK*-427s (3.1-fold) were observed. No major differences between the parent and mutant *sigF* transcript levels were observed at 12 and 14 h.

5.3.4 *sigK* is induced upon cold and hyperosmotic shocks and is required for optimal growth at low temperature and high salinity (V)

Significant induction of *sigK* expression was observed 5 h after both temperature downshift (2.8-fold) and hyperosmotic shock (1.9-fold), but not

upon pH downshift. No induction during optimal growth or upon entry to the stationary phase was observed.

The growth rates of *sigK*-427s and *sigK*-298as at 17 °C were markedly decreased from those of the wild type strain. Furthermore, the wild type strain reached significantly higher maximum optical density than either of the mutant strains. At 37 °C, the growth characteristics of all strains were essentially similar.

Under hyperosmotic conditions (4.5% w/v NaCl), the growth rates of both *sigK* mutants were slightly higher than that of the wild type. In contrast, the lag phases of both mutants were markedly increased when compared with the wild type. At pH 5.3, no significant differences in growth characteristics between the mutants and the wild type were observed.

6. DISCUSSION

6.1 Transcriptomic analysis of the cold shock response of *C. botulinum* (I)

The transcriptomic analysis of the cold-shock response of *C. botulinum* ATCC 3502 at 1 h and 5 h after temperature downshift revealed an induction of several metabolic pathways that had previously characterized cold-related functions in bacteria. Moreover, the induction of a large number of previously uncharacterized genes was also observed. A set of 16 genes was strongly induced already 1 h after the cold shock, indicating a specialized, acute cold shock response. However, all of these genes presented sustained and even further increased expression at the later stage of cold exposure. An important role for mechanisms encoded by the rapidly-induced genes, both in the rapid cold-shock response and in subsequent cold adaptation, is therefore likely. Furthermore, extensive metabolic remodeling was observed at the later stage of cold adaptation.

Of the induced genes, several had been functionally annotated as encoding mechanisms that are previously identified as being cold tolerance-related in bacteria. These included cold shock proteins (CSPs) (Phadtare, 2004; Schmid *et al.*, 2009; Söderholm *et al.*, 2011), a DEAD-box RNA helicase (Hunger *et al.*, 2006; Jarmoskaite & Russell, 2011; Markkula *et al.*, 2012b; Palonen *et al.*, 2012), and mechanisms involved in the uptake of compatible solutes (Ko *et al.*, 1994; Angelidis *et al.*, 2002; Hoffmann & Bremer, 2011). Apart from the previously characterized role of CSPs in cold tolerance of *C. botulinum* ATCC 3502 (Söderholm *et al.*, 2011), the full functionality and ultimate importance of these mechanisms in *C. botulinum* have yet to be characterized. However, the results suggest that those mechanisms that are already well characterized in other bacteria are also involved in the cold shock response and adaptation of *C. botulinum*.

Interestingly, the induction of several genes related to countering oxidative stress, maintaining cellular redox balance, and involved in iron storage and uptake were also observed upon cold shock. Similar induction of oxidative stress-related genes was observed upon cold shock in *B. subtilis* (Kaan *et al.*, 2002), and in cold growth in *L. monocytogenes* and *Psychrobacter arcticus* (Liu *et al.*, 2002; Bergholz *et al.*, 2009). Recently, the presence of secondary oxidative stress in a variety of stress conditions in *Bacillus spp.* was demonstrated (Höper *et al.*, 2005; Mols *et al.*, 2010; Mols & Abee, 2011a; Reder *et al.*, 2012a; den Besten *et al.*, 2013). Our observation on the induction of oxidative stress response upon cold shock strongly supports the hypothesis of secondary oxidative stress as a player in "general" stress. Therefore, the induction of the oxidative stress response to counter cold-induced secondary oxidative stress could play an important role in the

response to temperature downshift in *C. botulinum*.

Increased robustness and (cross-)protection against multiple environmental stressors, that arise from the exposure to sublethal stress has been demonstrated in bacteria (den Besten *et al.*, 2010a; den Besten *et al.*, 2013). Increased stress tolerance that arises from minimal food processing treatments presents challenges to the classical hurdle design principles used in food safety control. Therefore, the induction of the oxidative stress response upon cold stress is of special interest in the foodborne pathogen *C. botulinum*, and warrants further research.

Induction of several hitherto uncharacterized genes, that encode putative DNA-binding regulatory proteins, was observed upon cold shock. Inactivation of two of these genes (*cbo0477* and *cbo0558A*) resulted in an impaired cold tolerance, which suggests important roles for these regulators in the cold tolerance of *C. botulinum*. Moreover, homologs for these genes with similar genetic arrangements can be found across several *C. botulinum* genomes, which suggests the conservation of these mechanisms. This observation, together with the strong and sustained induction of these regulators (esp. of *cbo0558A*) upon cold stress highlights them as interesting candidates for biomarkers for cold-induced robustness (den Besten *et al.*, 2010a) in *C. botulinum*.

6.2 The role of the two-component system (TCS) CBO0366/CBO0365 in cold tolerance of *C. botulinum* (I-III)

Of the TCS-encoding genes predicted to be present in the *C. botulinum* ATCC 3502 genome (Wörner *et al.*, 2006; Sebaihia *et al.*, 2007), those encoding components of the TCS CBO0366/CBO0365 were the most strongly induced upon cold shock (I). Therefore, this mechanism was chosen for a more detailed study. The cold-induction was confirmed by RT-qPCR analysis. Furthermore, no induction was observed in the exponential growth phase at 37 °C or upon entry into the stationary phase, which confirmed the observed expression pattern to be cold-related, and thus not a stationary-phase event (II).

Insertional inactivation of either of the genes *cbo0366* or *cbo0365* resulted in significantly impaired cold tolerance, but did not affect growth at the optimal 37 °C (II). Furthermore, the overexpression of the CBO0366 sensor kinase in the wild-type strain increased the tolerance to low temperature, compared with that of the vector control (II). These observations suggest a central role for the CBO0366/CBO0365 TCS in response and adaptation to cold in *C. botulinum*.

Although the activity of TCSs is mostly controlled by a delicate balance of their phosphorylation state (Hoch, 2000; Mitrophanov & Groisman, 2008), our results suggest that increased synthesis of TCS components may be also

required for a sufficient response. The notion that the expression levels of both *cbo0366* and *cbo0365* were substantially elevated in cold, whereas down-regulation in cultures at optimal temperature was observed, supports the theory that increased amounts of TCS components might be required for an optimal cold stress response. Induced expression of TCS genes during cold stress has been reported for *B. subtilis* (Beckering *et al.*, 2002) and for *Y. pseudotuberculosis* (Palonen *et al.*, 2011). The improved cold tolerance of the strain that overexpressed CBO0366 (II) further supports the role of an increased TCS expression that helps the culture to adapt to low temperatures.

Further insight to the mechanisms regulated by the CBO0365 response regulator whereby the cold-sensitive phenotypes of the CBO0366/CBO0365 TCS mutants could be mediated was sought by a transcriptomic analysis. We compared transcriptomic profiles of the *cbo0365* response regulator mutant and the ATCC 3502 wild type at 37 °C and 15 °C (III). The expression of a large number of genes and operons was affected by the mutation. Furthermore, the direct transcriptional regulation of several affected genes and operons by CBO0365 was confirmed by electrophoretic mobility shift assays (III). The importance of components of the acetone-butanol-ethanol (ABE) fermentation, arsenic resistance, and phosphate uptake mechanisms in the cold tolerance response was demonstrated by impaired growth at 17 °C for mutants of genes that encode for the pathway components (III).

Bacteria counter cold-induced decrease in membrane fluidity by increasing the level of membrane fatty acid unsaturation (Suutari & Laakso, 1994; Zhang & Rock, 2008). While thoroughly characterized in *Escherichia coli* (Suutari & Laakso, 1994; Feng & Cronan, 2009), unsaturated fatty acid synthesis mechanisms in clostridia remain mostly unidentified (Zhu *et al.*, 2009). The *cbo3202*-encoded enzyme belongs to the crotonase superfamily, which harbors enzymes with diverse functions related to acyl-acyl carrier protein (ACP) and acyl-CoA modifications - the central steps in lipid biosynthesis (Hamed *et al.*, 2008). Thus, a possible explanation for the markedly cold-sensitive phenotype exhibited by the *cbo3202* mutant is that the putatively *cbo3202*-encoded 3-hydroxybutyryl-CoA dehydratase possesses an alternative function in fatty acid synthesis.

Another means for decreasing the membrane lipid melting point is to increase the proportion of branched-chain fatty acids (BCFA), especially *anteiso*-BCFA (Suutari & Laakso, 1994; Zhang & Rock, 2008). However, the mechanisms for generating branched-chain acyl-CoA primers for BCFA synthesis are unidentified in clostridia. Hypothetically, conversion of the central ABE fermentation acyl-CoA intermediates into structurally closely-related BCFA primers could serve as means to initiate BCFA synthesis in clostridia. Thus, the cold-sensitive phenotype observed in the *cbo3202* and *cbo3199* mutants could be attributed to the lack of putative BCFA synthesis precursors. Our data suggest an important role for the central ABE fermentation intermediates and the enzymes that catalyze their formation in cold tolerance of *C. botulinum*.

C. botulinum lacks the cold-induced O_2-dependent lipid desaturase system increasing membrane fluidity that was identified as a central cold stress-countering mechanism in *B. subtilis* (Aguilar *et al.*, 1998; Cybulski *et al.*, 2002; Altabe *et al.*, 2003). Therefore, other means to counter membrane solubilization in response to temperature downshift are possibly present. Disruption of the alcohol dehydrogenase-encoding *cbo1407* resulted in slower growth at 17 °C, and putatively results in hampered solvent (butanol and/or ethanol) formation. Butanol, which accumulates as an end-product of metabolism in solventogenic clostridia, has been shown to directly solibilize the lipid cell membranes (Vollherbst-Schneck *et al.*, 1984). It can thus be hypothesized, that the importance of intact solventogenic mechanisms at low temperatures could be attributed to the lipid-solubilizing effect of solvents, and subsequently to the reduced efficiency of rapid membrane adaptation in those mutants with impaired solventogenesis.

Among the significantly more expressed genes in the *cbo0365* mutant, the genes that putatively encode for arsenic resistance machinery were identified. In our previous studies, a possible correlation between arsenic resistance and tolerance towards low temperature was observed in *C. botulinum*. The Nordic (Group I) *C. botulinum* type B strains form two distinct clusters BI and BII (Nevas *et al.*, 2005a; Nevas *et al.*, 2005b; Lindström *et al.*, 2009). The cluster BI strains were shown to lack several arsenic resistance-related genes, including *arsR* (Lindström *et al.*, 2009). In contrast, all the investigated cluster BII strains harbored a full complement of the arsenic resistance machinery. As a result, cluster BI strains manifested markedly lower tolerance to sodium arsenite than cluster BII strains (Lindström *et al.*, 2009). Furthermore, significant differences in minimum and maximum growth temperatures were observed between the clusters, the arsenic-sensitive cluster BI strains were found to be less tolerant to low temperature than the cluster BII strains (Hinderink *et al.*, 2009). We investigated the apparent interconnection between cold tolerance and an intact arsenical resistance operon and its regulation further by comparing growth characteristics of *cbo0751* (*arsC*) and *cbo0753* (*arsR*) mutants at low temperature. Significantly impaired growth was indeed observed for the *cbo0751* (*arsC*) mutant at 17 °C compared to the wild-type strain and inactivation of *cbo0753* (*arsR*) completely abolished growth at 17 °C.

ArsR, together with ArsD, fine-tune the transcription levels of the *ars* operon (Wu & Rosen, 1993). Such a delicate regulation and ultimately arsenic resistance are prone to be imbalanced by disruption of any of the related regulators. Indeed, the inactivation of *arsC*, encoding the central detoxifying enzyme arsenate reductase, resulted in impaired tolerance to sodium arsenite. Moreover, the *cbo0365* and *arsR* mutants were both arsenic-sensitive, the latter presenting almost completely abolished growth in the presence of 0.1 mM sodium arsenite, whereas the wild-type strain was expectedly able to grow in these conditions (Lindström *et al.*, 2009). The growth defect was even more pronounced when the arsenic stress was

combined with cold stress: At 20 °C, the presence of sodium arsenite completely abolished the growth of the *cbo0365* mutant, while the mild cold stress alone allowed growth (II). Finally, recombinant CBO0365 was shown to bind to the promoter region of *cbo0753* (*arsR*), which is a finding that suggests the direct transcriptional control of the *ars* operon by CBO0365. These data support an important, novel role for an intact arsenical resistance operon, and more so for its undisturbed regulation, in robustness to low temperature in Group I *C. botulinum*.

A similarly increased expression in the *cbo0365* mutant as that found for the *ars* operon, was observed for the *pho* operon (*cbo2525-cbo2521*). The *pho* operon putatively encodes a phosphate uptake mechanism. Uptake of arsenic compounds into cells was shown to be facilitated by the *pho* operon-encoded phosphate transport system (Rosen & Liu, 2009). A role for this mechanism in attaining cold tolerance of *C. botulinum* was demonstrated by the deteriorated growth of the *cbo2525* mutant at 17 °C. The similar expression differences observed for both *pho* and *ars* operons suggest a regulatory link between the phosphate uptake and arsenic detoxifying mechanisms. This hypothesis is further supported by the binding of CBO0365 to putative promoters of both operons and thus direct control of expression by the CBO0366/CBO0365 TCS. How phosphate uptake, arsenical transport and detoxification ultimately are all related to clostridial cold tolerance remains unknown, however.

6.3 The role of the alternative sigma factor SigK in early-stage sporulation and in cold and hyperosmotic stress tolerance of *C. botulinum* (IV-V)

Both of the *sigK* mutants failed to produce viable, germinating spores after an anaerobic incubation period of 7 days. Such a time period was previously shown to be adequate for the efficient sporulation of clostridia to occur (Burns *et al.*, 2010). In stage 0 of sporulation, the cell elongates prior to asymmetric cell division typical for stage II. The *sigK* mutants exhibited elongated cells and no asymmetric cell division, characteristic of sporulation being disrupted at stage 0. Furthermore, the expression levels of *sigK*, *spo0A*, and *sigF* were observed to drop sharply upon entry to stationary phase, which suggested that early sporulation stage 0 was essentially over when the cultures entered the stationary phase. The results support the activity of SigK at the early stages of the sporulation cascade. Early-stage disruption of sporulation in a *sigK* mutant has been noted before for *C. perfringens* (Harry *et al.*, 2009).

Considerably lower *spo0A* and *sigF* expression levels were observed in the *sigK*-427s mutant than in the parent strain at late exponential and early stationary growth phases. These results provide further evidence that SigK

plays a crucial role in the early stages of sporulation in *C. botulinum*. The sporulation cascade in *Bacillus* spp. and clostridia commonly begins with the activation of Spo0A. The hypothesis, that SigK may directly or indirectly positively regulate *spo0A*, is supported by the finding that *sigK*, in addition to *spo0A* and *sigF*, is also transcribed in the late log growth phase. The transcription of *sigF* was reported to be driven by Spo0A-P (Hilbert & Piggot, 2004), thus the failure of the *sigK*-427s mutant to activate *spo0A* explains the lack of *sigF* induction in the mutant. The lack of *sigF* transcription indicates that this particular pathway had been blocked prior to stage II of the cascade: a phenomenon that is in agreement with the visual observation of the cells being blocked before asymmetric cell division occurs.

The results show an essential role of SigK in the early stages of sporulation in *C. botulinum* ATCC 3502, in contrast to its strictly late-stage role in *B. subtilis* and *C. difficile*. Moreover, the observed expression changes of *spo0A* and *sigF* in the *sigK* mutant, together with cell morphology characteristic of sporulation halted at stage 0, suggest some SigK-controlled expression of *spo0A*, and concurrently of *sigF*.

A general stress-responsive regulatory factor in a multitude of Gram-positive bacteria is the alternative sigma factor, SigB (Boylan *et al.*, 1993; Becker *et al.*, 1998; Chan *et al.*, 1998; Kazmierczak *et al.*, 2003). However, the genome of *C. botulinum* ATCC 3502 does not harbor a homolog for *sigB* (Sebaihia *et al.*, 2007), which suggests that mechanisms of general stress response in *C. botulinum* are different from those in the gram-positive model organism *B. subtilis*. Recent findings in *B. subtilis* suggest an interesting interconnection between the decisions to sporulate or to adapt to stress in a state of non-growing "vegetative dormant" cells (Reder *et al.*, 2012b; Reder *et al.*, 2012c). These observations have led to the proposition for a role for the stress sigma factor SigB in regulating the activity of Spo0A of *B. subtilis*. The lack of *sigB* in the *C. botulinum* ATCC 3502 genome suggests that the network of decision-making between sporulation initiation and stress adaptation of this organism is regulated differently to that proposed for *B. subtilis* (Reder *et al.*, 2012b; Reder *et al.*, 2012c). Thus, the behavior and role of SigK in the response to stress in *C. botulinum* ATCC 3502 was also investigated.

Although no induction for *sigK* was observed in the microarray analysis of the cold shock response (I), the RT-qPCR analysis did demonstrate significant induction for *sigK* upon temperature downshift (V). The expression levels of *sigK* reached a maximum of a threefold increase in the RT-qPCR experiment, thus the apparent lack of induction in the microarray experiment is probably due to different normalization procedures in the experiments. Insertional inactivation of *sigK* resulted in a growth defect at 17 °C when compared with the wild type strain (V). These findings suggest an important role for this alternative sigma factor in temperature downshift and in growth at low temperatures.

To investigate the role of SigK in other stress conditions, expression of *sigK* upon hyperosmotic and acid shocks was tested. Induction of *sigK* expression was observed upon exposure to hyperosmotic conditions i.e. sodium chloride, but not upon exposure to low pH. Inactivation of *sigK* caused increased lag times in culture medium with 4.5% NaCl, but did not affect the phenotype under acidic growth conditions. These observations suggest a role for SigK in response and adaptation to hyperosmotic stress, but not in response to low pH.

The *sigK* expression patterns we observed in the stress experiments were markedly different from the pattern observed under optimal growth conditions (IV-V). Therefore, the relatively rapid induction of *sigK* observed upon exposure to low temperature and hyperosmotic conditions can be concluded to be due to these environmental stresses and not a stationary phase event.

The mechanisms by which disruption of *sigK* results in reduced stress tolerance remain unknown. To date, no information about the role of SigK in stress tolerance of clostridia has been presented as far as we are aware. The lack of a recognized homolog of the general stress sigma factor SigB in *C. botulinum* suggests that the approach this organism has in coping with stress conditions differs considerably from SigB-possessing gram-positive bacteria. For instance, rather than integrating the general stress response network under one major regulator, several regulatory factors could be involved, and each of which has its own specific niche. The fact that *sigK* induction and phenotypic changes in *sigK*-inactivated strains were observed only under low temperature and hyperosmotic conditions, but not at low pH, may support this hypothesis. Our results suggest a stress-related alternative function for the hitherto strictly sporulation-associated sigma factor SigK of *C. botulinum*.

7. CONCLUSIONS

1. *C. botulinum* ATCC 3502 induces a relatively small set of genes as an acute response to cold shock. Extensive metabolic remodeling takes place after prolonged cold exposure. The cold-shock stimulon not only consists of mechanisms previously associated with bacterial cold tolerance, but also of several previously uncharacterized mechanisms. The results suggest a role for oxidative stress as a component of cold stress in this organism. Additionally, the cold-induced DNA-binding transcriptional regulators, CBO0477 and CBO0558A, play a role in the cold tolerance of *C. botulinum* ATCC 3502.

2. The CBO0366/CBO0365 TCS plays a central role in the tolerance of *C. botulinum* ATCC 3502 to to cold shock and its adaptation to low temperatures. This was demonstrated by the significantly impaired growth of both TCS mutants at 15 °C. Inactivation of *cbo0365* results in marked changes in the transcriptome of *C. botulinum* ATCC 3502, both at optimal and low temperatures. The TCS exerts cold tolerance via direct CBO0365-mediated transcriptional control of metabolic pathways that are related to acetone-butanol-ethanol fermentation, arsenic tolerance, and to phosphate uptake. The decreased cold tolerance of *C. botulinum* that results from the disruption of its ABE fermentation pathway could arise from impaired synthesis of precursors of branched-chain membrane lipids, or from blocked solvent formation that would hamper rapid solubilization of membrane lipids.

3. The alternative sigma factor SigK is crucial in early-stage sporulation in *C. botulinum* ATCC 3502, and at least partially regulates the activation of the sporulation master switch *spo0A*. Furthermore, SigK plays a significant role in the tolerance to cold and hyperosmotic stress but not to low pH stress in *C. botulinum* ATCC 3502, which suggests interconnections between sporulation and stress adaptation.

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